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"They did not know it was impossible so they did it"

- Mark Twain

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II. ABBREVIATION LIST

ADM	Acinar-to-Ductal Metaplasia	Hras	Harvey ras sarcoma
ADPKD	Autosomal dominant polycystic kidney disease	ICAM	Intercellular adhesion molecule 1
AP	Acute pancreatitis	IFT	Intraflagellar transport
BB	Basal body	IL	Interleukin
BRCA2	Breast cancer 2	IP3	Inositol triphosphate
CAC	Centroacinar cell	JAK	Janus Kinase
CCK	Cholecystokinin	Kras	Kirsten rat sarcoma
CDKN2	Cyclin-dependent kinase inhibitor 2A	MAPK	Mitogen-activated protein kinase
CK19	Cytokeratin 19	Mist1	Muscle, intestine, and stomach expression 1
COX2	Cyclooxygenase 2	MPC	Multipotent progenitor cell
CP	Chronic pancreatitis	NADPH	Nicotinamide adenine dinucleotide phosphate
EGFR	Epidermal growth factor receptor	NF- κ B	Nuclear factor-kappa B
ErbB	Erythroblastic leukemia viral oncogene homolog	Nkx6	Nkx6 homeobox factor
FAMMM	Familial atypical multiple mole melanoma	Nr5a2	Nuclear receptor subfamily 5, group A, member 2
GATA6	GATA binding protein 6	Nras	Neuroblastoma rat sarcoma
HNF	Hepatocyte nuclear factor	OC	Onecut

PanIN	Pancreatic Intraepithelial Neoplasia
PaSCs	Pancreatic stellate cells
PCM	Pericentriolar matrix
PDAC	Pancreatic ductal adenocarcinoma
Pdx1	Pancreatic and duodenal homeobox 1
PIP2	Phosphatidylinositol biphosphate
PKC	Protein kinase C
Prox1	Prospero homeobox protein 1
Ptf1a	Pancreas-specific transcription factor 1a
Rbpj	Recombining binding protein of hairless
ROS	Reactive oxygen species
Sox9	Sex Determining Region Y-related High Mobility Group box 9
SRY	Sex-determining region Y
Stat	Signal transducer and activator of transcription protein
TGF- β	Tumor growth factor- β

III. SUMMARY

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers. The exceptionally poor prognosis of this cancer is explained by the late detection which results from the lack of diagnostic symptoms at the early stages of the disease. Understanding the mechanisms initiating PDAC is necessary to accelerate diagnosis and improve patient survival and treatment. PDAC originates from acinar cells that undergo acinar-to-ductal metaplasia (ADM). This preneoplastic lesion can evolve into pancreatic intraepithelial neoplasia (PanIN) and further into invasive cancer. In this context, the aim of my thesis was to identify and characterize mechanisms that affect the maintenance of acinar cell differentiation.

We first showed that miRNAs are required for acquisition and maintenance of the differentiated state of acinar cells. The transcription factor Hnf6 is expressed in pancreatic progenitor cells and must be repressed to enable acinar cell differentiation. Using *in vitro* gain- and loss-of-function experiments and mouse knock-out models, we demonstrated that two miRNAs, namely miR-495 and let-7b, are required to repress Hnf6. Inhibition of these miRNAs are associated with ectopic expression of Hnf6 in acinar cells, leading to abnormal acinar differentiation in the embryo and ADM in adults. Therefore, we uncovered a miRNA-dependent transcriptional network which determines acquisition and maintenance of the acinar cell fate.

ADM in adult pancreas is also associated with ectopic expression of the transcription factor Sox9. Using mice deleted for Sox9 in acinar cells and *in vitro* experiments, we demonstrated that this transcription factor is required for ADM and PanIN development. Sox9 exerts this function by stimulating the activity of the ErbB pathway.

Finally, we showed that ADM can also result from defects originating in pancreatic duct cells. Mice with a ductal deletion of Hnf6 or of Liver kinase B1 (Lkb1) showed dilated pancreatic ducts, perturbed acinar cell differentiation, and ADM. In both mice, primary cilia of ductal cells were reduced in length and had abnormal morphology, suggesting a functional defect. Several mouse models, including those deficient in Hnf6 or Lkb1, show a tight association between ductal ciliary defects and pancreatitis with perturbed acinar homeostasis and differentiation. Therefore we suggest that perturbed homeostasis of the ducts, potentially driven by ciliary defects, leads to ADM.

In conclusion, our findings demonstrate that both differentiating and mature acinar cells are sensitive to cell-autonomous and tissular cues that can perturb their fate and induce ADM and neoplastic lesions.

IV. INTRODUCTION

A- Pancreas morphology and development

a. Pancreas histology and anatomy

The pancreas is a glandular organ located in the upper abdomen behind the stomach and close to the spleen. It is a mixed gland with endocrine and exocrine functions, which can be divided in three portions: the head, the body and the tail. The endocrine function regulates glucose and metabolic homeostasis by producing endocrine hormones. Endocrine cells form the so-called islets of Langerhans and secrete glucagon (α cells), insulin (β cells), pancreatic polypeptide (PP cells), somatostatin (δ cells) and ghrelin (ϵ cells) (Figure 1A-B). Endocrine cells represent 1-2% of the pancreatic cells and receive 10-15% of the pancreatic blood flow. Islets of Langerhans are equally located between the different parts of the rodent pancreas whereas in the human pancreas, endocrine cells are mainly found in the tail (Wang et al., 2013).

The exocrine part of the pancreas represents around 95% of the pancreatic cells and there is no difference in the content of exocrine cells between the different portions of the pancreas. Exocrine functions are exerted by the acinar cells, which produce digestive enzymes (peptidases, ribonucleases, deoxynucleases, amylases and lipases), required for bolus digestion, and by ductal cells which line the branched ducts that deliver the enzymes into the duodenum. Ductal cells produce bicarbonate that neutralizes stomach acidity and prevents intrapancreatic activation of acinar enzymes. Centroacinar cells (CAC) connect acinar cells to ductal cells (Figure 1B).

Histologically, acinar cells form units called acini. They consist of pyramidal epithelial cells with nuclei located at the basal pole and with granules of inactive enzymes (called zymogens) located at the apical portion of the cell. Apical portions

of acinar cells face a lumen and are connected to CAC. Each acinus is connected to an intercalated duct, which is lined by a cuboidal epithelium. These intercalated ducts join an intralobular duct which in turn is connected to an interlobular duct. Finally, interlobular ducts anastomose to the main pancreatic duct (Figure 1A-B).

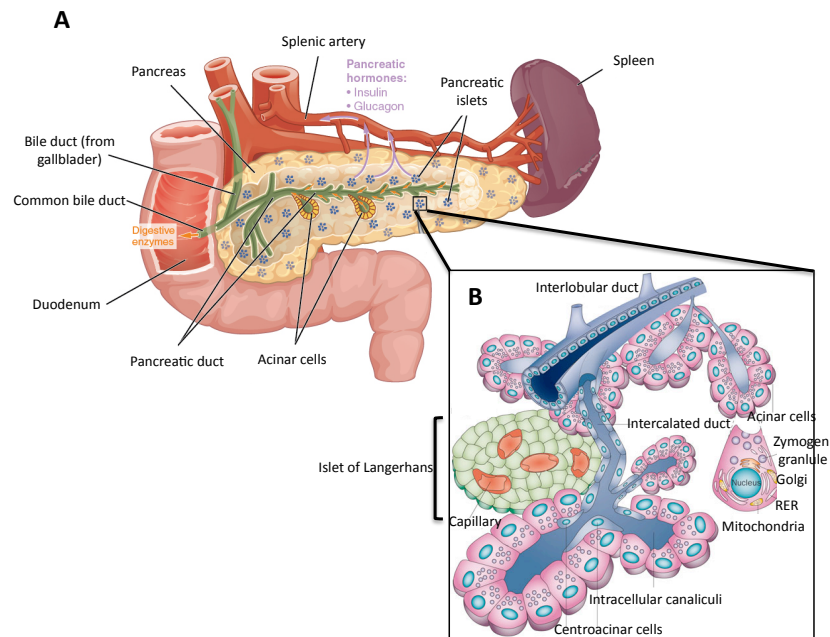


Figure 1. Anatomy of the pancreas (adapted from http://philschatz.com/anatomy-book/resources/1820_The_Pancreas.jpg and Logsdon et al., 2013). **(A)** Schematic structure of the pancreas and **(B)** magnification showing the organization of the different pancreatic cell types. See text for additional details.

Food intake induces morphologic changes in acinar cells. Number and size of zymogen granules decrease as they are released in the ducts. Release is mediated by cholecystikine (CCK), a hormone produced by cells located in the duodenum and proximal jejunum (Wang et al., 2007). The most potent stimulants of CCK secretion are fats and proteins, leading to secretion of CCK into the circulation to target CCK receptors on the acinar cells. Activation of digestive

enzymes occurs in the gut when the environment becomes acidic and by conversion of trypsinogen into trypsin by the intestinal brush-border protease enterokinase.

A mesenchyme-derived stroma of loose connective tissue surrounds the pancreas and divides it into lobules. In normal condition, the stroma represents less than 1% of the pancreas. It is composed of endothelial cells and quiescent fibroblasts. Complex interactions between epithelial and stromal cells support the normal physiology and homeostasis of the pancreas (Rhim and Stanger 2010). Following injury of the pancreas, the stromal compartment is activated and plays role in fibrogenic and carcinogenic processes. Pancreatic stellate cells (PaSCs), a specialized fibroblast type, were found to produce extracellular matrix when activated and there is compelling *in vitro* and *in vivo* evidence of PaSCs' contribution to the progression of chronic pancreatitis and pancreatic cancer development (Masamune et al. 2009).

Loss of pancreatic homeostasis is associated with perturbed maintenance of endocrine and exocrine cells. Endocrine perturbations lead to diabetes type 1 or 2 as well as pancreatic neuroendocrine tumours. Homeostasis of exocrine compartment can be disrupted following injury such as alcohol abuse or smoking. These conditions lead to pancreas inflammation and reactivation of signaling pathways active during pancreas development. Sometimes these changes culminate in the emergence of a malignant process. The number of diseases that result from pancreatic dysfunction underscores the need to understand embryonic molecular pathways to figure out key players in exocrine disease development. For this reason, the next chapter will give a short overview on pancreas development and will summarize the key steps of acinar and ductal differentiation.

b. Pancreas development

The first sign of pancreas development appears at embryonic day (e) 9 in mice and on the 26th day of gestation in humans as the endoderm, at the foregut-midgut junction, forms a dorsal bud and a ventral bud which rapidly evaginate into the overlying mesenchyme. This budding process creates a multilayered, pseudostratified epithelium in which microlumens appear around e11 (Figure 2). It has been shown that microlumen formation initiates duct development (Kesavan et al. 2009). Dorsal and ventral buds fuse together around e12.5 in the mouse, and e37 to e42 in humans (Gittes 2009). During these phases, highly proliferative multipotent progenitor cells (MPCs) give rise to differentiating acinar, endocrine, and ductal cells. These undifferentiated progenitors are located within growing branch tips (Figure 2).

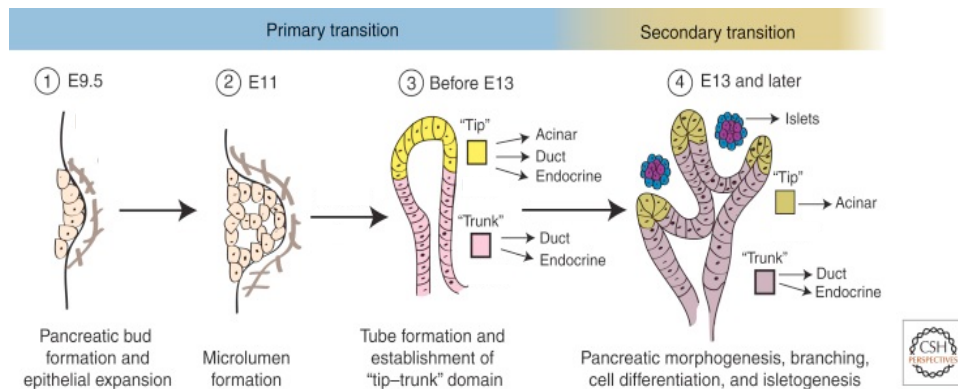


Figure 2. Pancreatic morphogenesis and developmental regulation (Benitez et al. 2012).

At approximately e13 to e14 in the mouse, dramatic changes (associated with the so-called secondary transition step) occur in the cellular architecture of the pancreas. Rapid branching morphogenesis occurs and microlumen structures coalesce to form the continuous, single-lumen ductal system characteristic of pancreas morphology (Hick et al. 2009, Villasenor et al. 2010). The epithelial architecture is composed of two different cell compartments reminiscent of a tree: epithelial tips and trunks. The tips divide rapidly and give rise to acinar cells which will rapidly form zymogen granules and express acinar-specific genes. The trunk cells give rise to differentiated ducts and, by delamination from the tubular epithelium, to the endocrine cells (Figure 2). MPCs are progressively depleted from the pancreas, in parallel to the acquisition of the mature pancreatic architecture (Zhou et al. 2007, Solar et al. 2009). From e18.5 onwards, most trunk cells develop into duct cells, although until 3 weeks after birth, duct cells continue to undergo non- β endocrine cell neogenesis at a low rate (Kopp et al. 2011).

1. *Acinar differentiation*

Differentiation of exocrine pancreatic cell types requires many transcription factors that act in a precise spatiotemporal cascade (Cleveland et al. 2012). Histologically distinct acinar and ductal cells do not appear before approximately e14. The transcription factor Ptf1a is critical for acinar cell differentiation, absence of Ptf1a resulting in failure of acinar cell formation (Krapp et al. 1998). During pancreas development, cross-repressive interactions between transcription factors Nkx6.1/Nkx6.2 (Nkx6) and Ptf1a are needed to specify fate choice of MPCs into ductal/endocrine and acinar cell fate, respectively. The transcription factor Prox1 may also cooperate with Nkx6.1 in this specification step (Figure 3) (Schaffer et al. 2010).

Ptf1a/P48 is a basic helix-loop-helix (bHLH) DNA-binding protein which is part of the Pancreas Transcription Factor 1 (PTF1) complex with two other DNA-binding proteins, Recombining binding protein suppressor of hairless (Rbpj)/RbpjL

and a class A basic helix-loop-helix (bHLH) protein (for example E12 or E47). PTF1 is the master transcriptional regulator and activator of digestive enzyme genes through its binding sites in their promoter. In acinar precursor cells, the PTF1-J complex binds and activates the gene encoding a pancreas-restricted Rbpj paralog, Rbpjl (Masui et al. 2008, Masui et al. 2010). From e13 to e15, Rbpjl progressively replaces Rbpj in the PTF1-J complex to form the PTF1-L complex, which binds and activates the acinar cell-specific digestive enzyme genes. The PTF1-L complex is essential to ensure a mature acinar phenotype in adult pancreas, as PTF1-L is responsible for the acinar cell-specific expression of most of acinar genes. The nuclear receptor Nr5a2 has also been shown to interact physically with Ptf1a and to activate expression of digestive zymogen genes and acinar cell-specific genes (Figure 3) (Cleveland et al. 2012). Another transcription factor, Mist1, a bHLH protein, is not required for the initial specification of the exocrine pancreatic lineage but is essential for establishment of the secretory machinery and a fully mature acinar phenotype (Pin et al. 2001).

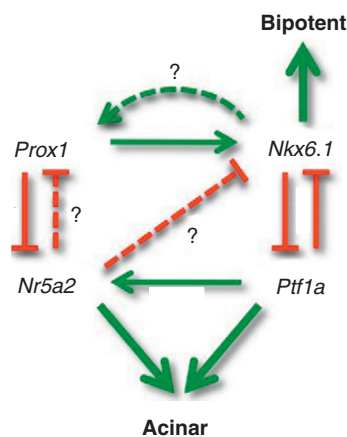


Figure 3. Regulatory interactions among transcription factors involved in the exocrine fate choice (MacDonald et al. 2010). Ptf1a and Nkx6.1 mutually antagonize each other's expression leading to endocrine/duct or acinar fate. Similar relations exist between Prox1/Nr5a2. Dashed lines indicate lack of experimental evidence.

2. Ductal differentiation

As discussed above, the trunks contain both a central duct-like epithelium and adhering cells that have initiated endocrine differentiation and that will rapidly delaminate from the tubular epithelium. Multiple transcription factors contribute to the duct/endocrine fate. The homeodomain transcription factor Pdx1, one of the first genes expressed at the onset of pancreas development, is still expressed at high levels between embryonic day e9.5 and e12.5 in the vast majority of duct progenitors (Ahlgren et al. 1996, Gu et al. 2002). At this stage, Pdx1 is required for branching morphogenesis (Wescott et al. 2009).

Besides Pdx1, three other transcription factors are also important for duct development. Sex-determining Region Y-related HMG box transcription factor 9 (Sox9) is a key factor for pancreas development and differentiation, which is expressed in progenitor cells and prevents their premature differentiation. Sox9 conditional knock-out (KO) mice show severe pancreatic hypoplasia resulting from depletion of the pancreatic progenitor pool (Seymour et al. 2007). Later, endocrine precursors arise from Sox9⁺ cells, demonstrating requirement of Sox9 for specification of an endocrine fate (Seymour et al. 2008). Moreover, Sox9 is required for ductal cell differentiation and maintenance. Indeed, when Sox9 is inactivated, ductal cells fail to differentiate leading to formation of polycystic ducts devoid of primary cilia (Shih et al. 2012), a membrane-bound organelle important for the modulation of signaling pathways (see chapter C of this introduction for details). Genetic lineage tracing experiments have shown that Sox9⁺ cells give rise to cells of the different pancreatic lineages during embryogenesis, but in adult, no acinar or endocrine cells arise from Sox9⁺ ductal cells (Kopp et al. 2011).

Another transcription factor critical for pancreatic ductal development is Hepatocyte Nuclear Factor 1 β (HNF1 β /Tcf2). In pancreas lacking Hnf1 β , Ptf1a expression is not induced leading to agenesis of the ventral pancreas and rudimentary development of the dorsal pancreas (Haumaitre et al. 2005). During the secondary transition, Hnf1 β expression is restricted to the pancreatic trunk and

later to the adult ductal cells. Hnf1 β conditional KO mice show a cystic phenotype with alterations in acquisition of ductal features. This demonstrates a crucial role for HNF1 β in the control of early and late duct morphogenesis. HNF1 β performs this function by controlling expression of genes involved in cystogenesis and duct formation (De Vas et al. 2015).

Finally, our lab has a long standing-interest in a transcription factor called Hepatocyte Nuclear Factor 6 (Hnf6/Onecut1), which is important for pancreatic development. Initially, Hnf6 cDNA was isolated and characterized as a factor belonging to the new ONECUT (OC) class of cut homeodomain proteins. This class is composed of two other members, OC2 and OC3, in mammals, and is characterized by a bipartite DNA-binding domain constituted of a single cut domain and a divergent homeodomain. This homeodomain differs from others by the presence of two amino acids, a phenylalanine at position 48, and a methionine at position 50, which are never found at these positions in any other homeodomain proteins (Lemaigre et al. 1996, Lannoy et al. 1998) (Figure 4). Despite extensive homology between the different OC factors, no redundancy between Hnf6 and OC-3 is found during pancreatic morphogenesis. In contrast, Hnf6 and OC2 show partially redundant functions during endocrine differentiation as double Hnf6/ OC2 null mice show a more severe endocrine deficiency than Hnf6-null mice (Vanhorenbeeck et al. 2007).



Figure 4. Schematic representation of transcription factor Hnf6 (from http://sites.uclouvain.be/nedi/NEDI/Laboratoire_NEDI_-_Onecut.html). The DNA binding domain is displayed in blue. Green boxes/residues show activator domains of transcriptional activity and red boxes, transcriptional repressor domains.

During development, Hnf6 is expressed as early as e8 at the foregut-midgut junction and its expression in the early endoderm is controlled by Hnf1 β (Landry et al. 1997, Poll et al. 2006). Hnf6 is required for timely initiation of Pdx1 expression in the endoderm. Accordingly, Hnf6-null mice show hypoplasia of the pancreas due to a delayed onset of Pdx1 expression (Jacquemin et al. 2003). Most of Hnf6 KO mice die between postnatal day 1 and 10 probably due to a liver failure, but 25% reach adulthood. The latter show impaired endocrine differentiation; this role in endocrine cell specification is explained by the fact that Hnf6 controls the expression of Ngn3, a transcription factor essential for the formation of the endocrine precursor cells (Jacquemin et al. 2000). Late in gestation, downregulation of Hnf6 expression in endocrine cells is essential for β -cell maturation and islet morphogenesis (Gannon et al. 2000).

In addition to its role in endocrine differentiation, Hnf6 is also required for exocrine duct differentiation. Hnf6 is expressed in the pancreatic duct epithelium throughout development, and loss of Hnf6 leads to the formation of cystic ducts devoid of primary cilia in inter- and intralobular ducts but not in intercalated ducts (Pierreux et al. 2006). By regulating genes involved in tubulogenesis and polycystic diseases in a similar way to Hnf1 β , Hnf6 is an important actor of pancreatic duct development (Maestro et al. 2003, Pierreux et al. 2006). Mice with a conditional deletion of Hnf6 initiated during pancreas development show the same endocrine and ductal defects than those found in mice with a constitutive Hnf6 deletion. In addition, the former show acinar perturbation; thus, pancreatitis, a well-known risk factor for pancreatic cancer, is observed in these mice (Zhang et al. 2009). All together, these results indicate that Hnf6 is a critical player of endocrine and exocrine development.

B- Pancreatitis, preneoplastic and neoplastic lesions, and PDAC development

a. Pancreatitis pathophysiology

Pancreatitis is an inflammation of the pancreas. Usually it results from premature activation of zymogens within the pancreas. This activation leads to damage of the organ with necrosis and apoptosis of the tissue and elevation of serum levels of pancreatic enzymes. This is one of the most prevalent disorders of the exocrine pancreas and can occur in an acute or chronic condition. Pancreatitis is also one of the best-established risk factor for pancreatic ductal adenocarcinoma (PDAC).

1. *Acute Pancreatitis*

Acute pancreatitis (AP) is a sudden inflammation of the pancreas characterized by tissue edema, acinar cell necrosis and apoptosis, hemorrhage and inflammation. It is one of the most frequent gastrointestinal causes of hospital admission in the USA. Acute pancreatitis is classified as mild, moderate or severe based on physiological parameters and clinical chemical changes in the serum and urine of the patients. Although most episodes are classified as mild and damages are usually reversible, the overall mortality remains 5 to 10% owing to complications such as systemic inflammatory response syndrome and multi-organ dysfunction syndrome (Hegyi et al. 2011). Knowledge of AP has led to better therapeutic approaches but simultaneous activation of multiple biological pathways raises difficulties in development of effective treatments. Obstruction of pancreatic ducts by gallstones is the most common cause of AP, and women and the elderly are at higher risk (Yadav and Lowenfels 2006). Alcohol abuse is the second most common cause of AP and is known to increase the risk of pancreatitis by boosting the sensitivity of the pancreas to injury from other factors such as environmental or genetic factors (Pandol et al. 2011). The risk of developing AP is linked to the

amount and duration of alcohol consumption; it ranges from 2% to 5% among people who are heavy and regular consumers of alcohol (Yadav and Lowenfels 2013). Recently a link between AP and PDAC has been demonstrated. Patients older than 40 with a previous episode of AP have an increased risk of developing PDAC and the risk is greater the first year after an AP episode but subsequently decreases rapidly (Munigala et al. 2014).

2. Chronic Pancreatitis

Chronic pancreatitis (CP) is a disease that results in permanent morphological changes in the pancreas with chronic inflammation and fibrosis. This leads to impairment of exocrine and endocrine functions by destruction of pancreatic tissue and replacement with fibrotic tissue. Despite advances in the comprehension of the pathogenesis, no curative treatment currently exists. Much of our knowledge of CP derives from the hereditary form of pancreatitis which is histologically very similar to the non-hereditary form of CP. Annual incidence of CP ranges from 5 to 12/100.000 persons and is lower than AP but patient quality of life is more affected by this form of pancreatitis (Yadav and Lowenfels 2013). Usually CP patients present indigestion problems and abdominal pain, management of which is the main therapeutic challenge. Regular smoking and alcohol consumption are common causes for CP and frequently coexist, although they also appear as independent risk factors (Talamini et al. 1999). Alcohol alone is responsible for more than 70% of cases. Usually 6 to 12 years of alcohol misuse generate CP after relapsing episodes of AP (Gupta and Toskes 2005). However, for unknown reasons, one single severe episode of AP can sometimes lead to a chronic disease. Besides idiopathic cases, known causes for CP development are genetic mutations and cystic fibrosis. Numerous studies demonstrated a strong link between antecedent CP and increased risk to develop PDAC. Patients with CP have a 13-fold higher risk of developing PDAC (Yadav and Lowenfels 2013, Zambirinis et al. 2014).

3. *Rodent models of caerulein induced-pancreatitis*

To better understand underlying mechanisms of pancreatitis, to improve therapy, and to potentially prevent development of PDAC, rodent models of pancreatitis have been created. Several models for acute and chronic pancreatitis resembling the human situation have been developed. Most pancreatitis models use mice or rats, because they are commonly used as laboratory animals, are less expensive to raise and better standardized than other animal models. However it is important to keep in mind that none of these models perfectly mimics the pathophysiology of human pancreatitis. In this chapter, we will only describe the most popular model that resorts to a peptide called caerulein to induce pancreatitis (Hyun and Lee 2014). This model was quickly adopted because of its lack of requirement for surgery or complicated manipulations, and the easy way to deliver caerulein intraperitoneally. Other models include pancreatic duct ligation, alcohol injections, diet, or genetic mutations.

Caerulein is a decapeptide originally obtained from the skin of *Hyla caerulea*, an Australian frog. It is an analog of the hormone cholecystokinin (CCK) which is secreted by gastrointestinal cells following food intake. CCK exerts its effects on CCK receptors, which are located at the basolateral membrane of acinar cells where it stimulates secretion of enzymes, and, of the gallbladder cells where it induces contraction. There are two types of CCK receptors, CCK-1 and CCK-2, CCK-1 receptors being predominant in rodent pancreas. They are classical seven-helix membrane spanning G-protein coupled receptors which show different binding affinity for CCK (Kim 2008, Smith and Solomon 2014, Konno et al. 2015). CCK binding to its receptor activates phospholipase C. Then, activated phospholipase C hydrolyses phosphatidylinositol biphosphate (PIP₂) to generate inositol triphosphate (IP₃) and diacylglycerol. IP₃ binds to an IP₃ receptor located on the endoplasmic reticulum membrane (ER). This receptor serves as a Ca⁺⁺ channel; upon IP₃ binding, Ca⁺⁺ is released from the endoplasmic reticulum into the cytoplasm of acinar cells. Increased cytoplasmic concentration of Ca⁺⁺ and

diacylglycerol leads to activation of protein kinase C (PKC) and to initiation of diverse signalling pathways involved in enzyme secretion, increased protein synthesis, and cellular proliferation (Aghdassi et al. 2011, Smith and Solomon 2014). When CCK or caerulein is injected at supramaximal doses, it generates a distinct response that induces reduced apical enzyme secretion by acinar cells and further accumulation of zymogens in the cytoplasm which induces their basolateral secretion. This abnormal secretion leads to their intrapancreatic activation, which ultimately leads to pancreatic injury (Gorelick and Thrower 2009). Pancreatic injury depends on the caerulein concentration, number of injections, and duration of the study. By treating mice for shorter or longer periods, this model allows to study the development of both AP and CP (Figure 5).

At supramaximal doses of caerulein, Ca^{++} activates PKC-dependent phosphorylation of phospholipase A2, which is necessary for the production of arachidonic acid and subsequent activation of NADPH oxidase. Following activation, reactive oxygen species (ROS), which are important mediators of pancreatitis initiation, are produced. By directly activating NF- κ B, ROS contribute to cytokine production by acinar cells (Kim 2008, Morgan and Liu 2011). ROS also play a role as chemoattractant for inflammatory cells and promote inflammatory process. Inflammatory infiltration is one of the main characteristics of human and experimental pancreatitis. Activation of NF- κ B leads to expression of intercellular adhesion molecule-1 (ICAM-1), a cell surface glycoprotein which mediates neutrophil adhesion to the pancreas. Caerulein injection and subsequent ROS production also lead to activation of the Jak2/Stat3 pathway which induces secretion of IL-1 β and attraction of inflammatory cells (Figure 5) (Zaninovic et al. 2000).

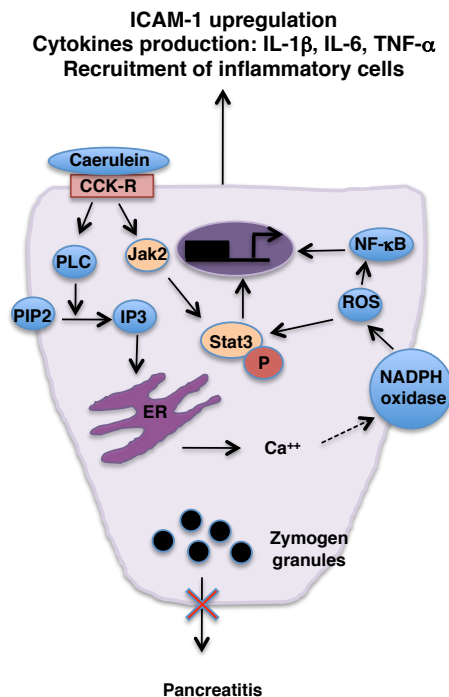


Figure 5. Tentative mechanism of caerulein-induced effects on acinar cells. Caerulein binds to the CCK receptor. This leads to activation of NF- κ B and Jak2/Stat3 pathways with upregulation of ICAM-1 and cytokine production by acinar cells.

Neutrophils are one of these inflammatory cell types. During AP, neutrophils have been proposed to play an important role in pancreatitis initiation by contributing to activation of trypsinogen. Neutrophils express high levels of NADPH oxidase and chemical reactions catalysed by this enzyme generate products which could regulate trypsin activation in the pancreatic tissue (Gukovskaya et al. 2002, Abdulla et al. 2011). It has been demonstrated that trypsin activation is important for initiation of AP but not CP. Indeed constitutively active trypsin in acinar cells is not sufficient to induce CP (Gaiser et al. 2011). In mouse models carrying a mutation enhancing autoactivation of trypsinogen, no CP develops even if these mice are more susceptible to caerulein-induced pancreatitis (Weiss et al. 2008). The principal mechanism necessary for the development of CP

seems to be persistence of NF- κ B activation after caerulein injections. As explained above, increased levels of NF- κ B activation are found in acinar cells after supramaxial doses of caerulein, leading to overexpression of another key mediator of chronic inflammation, COX2 (Daniluk et al. 2012). Consequently, sustained activation of NF- κ B may be a crucial mechanism for CP (Sah et al. 2013). This activation of NF- κ B generates production of inflammatory cytokines such as IL-1 β , IL-6 and TNF- α which attract monocytes and macrophages. In turn, these inflammatory cells also produce cytokines and chemokines. Consequently, more and more leukocytes are recruited and the inflammatory response increases. If the injury is recurrent, this leads to activation of PaSCs. Activated PaSCs generate collagen deposits leading to pancreatic fibrosis, a feature of CP (Figure 6).

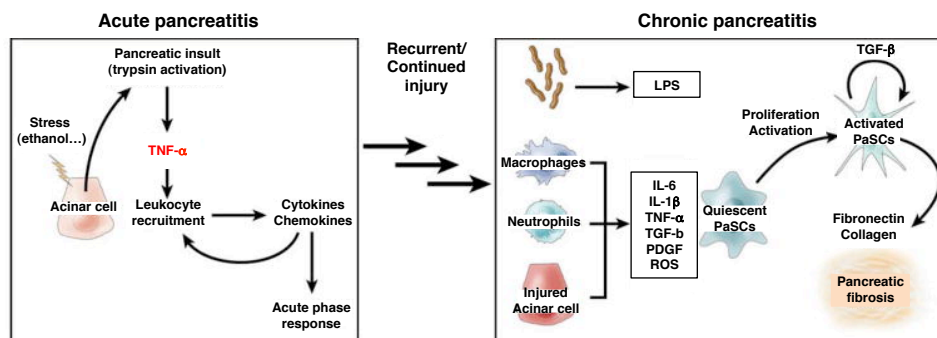


Figure 6. Immune cell involvement in acute and chronic pancreatitis (Zheng et al., 2013). During acute pancreatitis, injured acinar cells attract leukocytes and this leads to an acute phase response. After recurrent/continued injury, CP can develop, and generation of inflammatory cytokines induces activation of PaSCs and fibrosis.

b. Acinar-to-Ductal Metaplasia

During pancreatitis, acinar cells can undergo acinar-to-ductal metaplasia (ADM), which modifies acinar cells into duct-like cells. This process is characterized by a decrease of acinar genes (Ptf1a, Mist1, digestive enzymes...), and expression of ductal and embryonic genes, such as Hnf6, Sox9 and Pdx1, within the injured acinar cells (Hall and Lemoine 1992, Prevot et al. 2012). Histologically metaplastic acinar cells are cuboidal or flattened, and metaplastic acini present an enlarged lumen (Figure 7).

Metaplasia is frequently associated with an increased risk of subsequent neoplasia and cancer. Consequently ADM can be considered a precursor lesion of PDAC. Understanding how these lesions arise could help to prevent PDAC. For a long time PDAC has been thought to arise from ductal cells based on its morphologic characteristics. However the study of genetically engineered mouse models has demonstrated that acinar cells can transdifferentiate into duct-like cells and then evolve into neoplastic lesions (Kopp et al. 2012). Recently, it was demonstrated that human acinar cells also have the potential to undergo ADM (Houbracken et al. 2011).

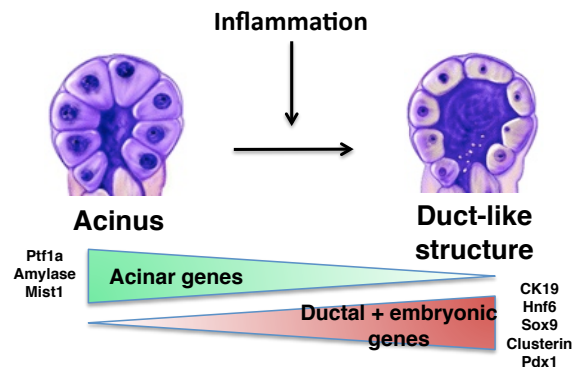


Figure 7. Schematic representation of acinar-to-ductal metaplasia. Mature acinar cells express acinar markers such as Ptf1a, amylase or Mist1. When inflammation occurs, acinar cells start to transdifferentiate into duct-like cells. Tubular complexes showing enlarged lumen are formed. Metaplastic acinar cells in these complexes express ductal markers like CK19, Hnf6 and Sox9 and re-express the embryonic markers clusterin and Pdx1.

The metaplastic conversion of acinar cells can also be modeled by *in vitro* 3D culture systems, which have been used to bypass the difficulty of studying metaplastic formations *in vivo*. When acinar cells are cultured in, or on, adequate matrix, loss of acinar cells is associated with increase of ductal structures (De Lisle and Logsdon 1990, Githens et al. 1994, Rooman et al. 2000). It was demonstrated that transdifferentiation occurs via a dedifferentiated intermediate cell type expressing the embryonic marker nestin. This intermediate cell concomitantly expresses acinar and ductal markers, before the complete loss of acinar genes, when metaplasia progresses (Means et al. 2005).

It has been suggested that these acinar-to-ductal changes are needed to protect acinar cells against pancreas injury such as caerulein-induced pancreatitis and to promote a regenerative response. It is well recognized that caerulein-induced pancreatitis is a fully reversible process. Following caerulein injections, most of acinar cells undergo apoptosis. However, in the surviving acinar cells, a powerful regenerative response occurs leading to full restoration of pancreatic functions within a week, with acinar proliferation along a redifferentiation process. During this regenerative process, surviving acinar cells re-activate embryonic

pathways. Notch signaling is important during pancreas development to prevent premature differentiation of the progenitor cell population. When regeneration occurs, Notch signaling is reactivated and deletion of this pathway leads to impaired regeneration of the pancreas (Jensen et al. 2005, Siveke et al. 2008). In an *in vitro* model of ADM, overexpression of Notch components such as the intracellular domain of Notch1 (Notch1-ICD) and Notch2-ICD in acinar cells leads to an undifferentiated intermediate expressing the marker nestin (Miyamoto et al. 2003). It has also been demonstrated that Notch interacts with β -catenin to modulate its activity, and β -catenin is required for proliferation of acinar cells during regeneration as deletion of the β -catenin gene dramatically delays recovery from caerulein-induced pancreatitis (Siveke et al. 2008, Morris et al. 2010, Keefe et al. 2012). Another embryonic pathway reactivated during ADM is the Hedgehog (Hh) signaling. During the regenerative process, Hh seems to be necessary for the redifferentiation process as suppression of this pathway during caerulein-induced pancreatitis leads to formation of metaplastic structures that fail to redifferentiate into acinar cells (Fendrich et al. 2008) (Figure 8).

Finally it has been shown that the Epidermal Growth Factor Receptor (EGFR) signaling pathway is implicated in ADM. It is one of the most important pathways for growth, survival and proliferation. Four receptors have been identified, namely Epidermal Growth Factor Receptor (EGFR), v-erb-b2 erythroblastic leukemia viral oncogene homolog (ErbB2)/Human Epidermal Growth Factor Receptor 2 (HER2), ErbB3 and ErbB4, which are cell-surface receptor tyrosine kinases. Ligand binding induces homo- or hetero-dimerization of the receptors; an exception is ErbB2 that does not bind any ligand even if it is able to dimerize. Dimerization stimulates a tyrosine kinase activity located in the cytoplasmic portion of the receptor (absent from ErbB3). Numerous ligands with an EGF-like domain activate the EGFR pathway (Jones et al. 1999). Many studies have shown the importance of this pathway for ADM development. A well-known ADM inducer is the ErbB ligand Transforming Growth Factor- α (TGF α). Mice expressing TGF α under the control of the metallothionein 1 promoter, exhibit fibrosis and pancreatic metaplasia (Jhappan et al. 1990, Song et al. 1999). TGF α

also induces ADM *in vitro* in cultured acinar cells. When EGFR/ErbB2 tyrosine kinase inhibitors are added in the culture medium, metaplastic lesions decrease in a dose-dependent manner, showing the requirement of EGFR signaling for the generation of ADM. The same conclusion is obtained with acinar cultures from mice with deleted EGFR alleles (Means et al. 2005, Navas et al. 2012).

In addition to signaling pathways necessary for the metaplastic/regenerative process, transcription factors controlling ADM have been identified. The acinar transcription factor Mist1 regulates adult pancreas homeostasis and locks acinar cells into a fully differentiated phenotype. Mice with a constitutive deletion of Mist1 develop a phenotype close to that caused by supramaximal doses of caerulein, with development of metaplastic lesions, activation of PaSCs, and defects in the exocytosis pathway (Pin et al. 2001, Zhu et al. 2004). Following AP induction, the phenotype of these mice is more severe, with a longer recovery period (Kowalik et al. 2007). Surprisingly, sustained expression of Mist1 leads to a dramatic increase in pancreatic damage and in acinar cell death (Karki et al. 2015). Together, these results indicate that reducing Mist1 expression is initially critical for acinar cells to survive pancreatitis: this probably allows acinar cells to suppress their secretory function and opens a window of cell proliferation. Subsequently, Mist1 has to be reactivated to fully re-establish functional acinar cells (Karki et al. 2015) (Figure 8).

The transcription factor Ptf1a is also involved in maintenance of acinar phenotype (Masui et al. 2010). When Ptf1a is deleted in adult acinar cells, ADM develops throughout the pancreas and pancreatic epithelial cells proliferate. Thus downregulation of Ptf1a is a decisive step in the process of ADM, showing its critical role to maintain acinar cell differentiation and prevent metaplastic transformation (Krah et al. 2015) (Figure 8).

Recently, the transcription factor Nr5a2 also called LRH-1 was identified as a susceptibility locus for PDAC (Amundadottir et al. 2009, Rizzato et al. 2011). Nr5a2 is normally downregulated during the metaplastic process (Figure 8), and in a mouse model of Nr5a2 deletion, Nr5a2-deficient mice demonstrated loss of

acinar cell identity; this phenotype is potentially linked to the interaction of Nr5a2 with the PTF1 complex and the importance of this interaction in the control of acinar gene expression. When pancreatitis is induced by caerulein injections, mice lacking Nr5a2 show no regeneration over the time, indicating that Nr5a2 is required for effective regeneration of acinar cells after pancreatic injuries (von Figura et al. 2014).

Finally, Gata6 has been demonstrated to be required for acinar fate maintenance and regulation of digestive enzymes by binding to the RbpjL and Mist1 promoters. Mice lacking Gata6 in the pancreas also show signs of ADM (Martinelli et al. 2013).

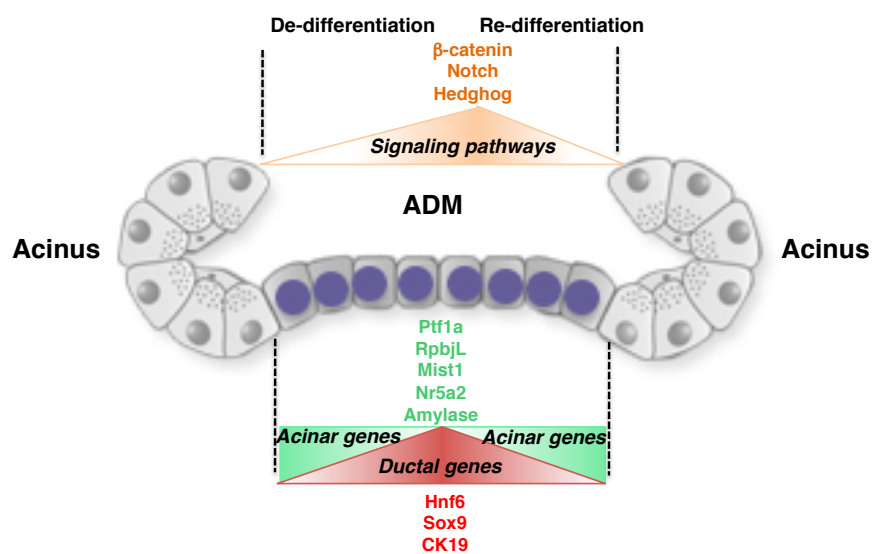


Figure 8: Schematic representation of ADM and redifferentiation processes. Following injury mature acinar cells start to downregulate acinar genes and to upregulate ductal genes. During the redifferentiation process, Nr5a2, Ptf1a and Mist1 are necessary to fully reestablish functional acinar cells as well as Wnt, Notch and Hedghog signaling pathways.

c. Pancreatic Intraepithelial Neoplasia

As described above, ADM is a reversible process supported by the powerful regenerative capacity of the acinar cells following caerulein-induced pancreatitis. However some mutations lock acinar cells into a duct-like phenotype. In association with a persistent inflammation, these mutations induce the emergence of Pancreatic Intraepithelial Neoplasia (PanIN) and eventually invasive pancreatic adenocarcinoma. PanIN are microscopic lesions (< 5 mm) undetectable by medical imaging. They are classified into PanIN-1A, -1B, -2 and -3, depending on their histology. PanIN-1A are flat epithelial lesions composed of tall columnar mucin-containing cells with nuclei located at the basal pole. Usually there is no nuclear atypia at this stage. When neoplastic lesions acquire a papillary, micropapillary or basally pseudostratified architecture, they are classified as grade 1B. At the PanIN-2 grade, nuclear abnormalities like enlarged nuclei, nuclear crowding or nuclear hyperchromatism are seen. These lesions can be flat or papillary. PanIN-3 stage is reached when cribriform luminal necrosis and cytologic abnormalities are seen. Additionally, small clusters of epithelial cells bud off into the PanIN lumen and severe nuclear atypia are observed with loss of polarity. PanIN-3 is called “carcinoma in situ” and is the stage preceding invasive carcinoma (Figure 9).

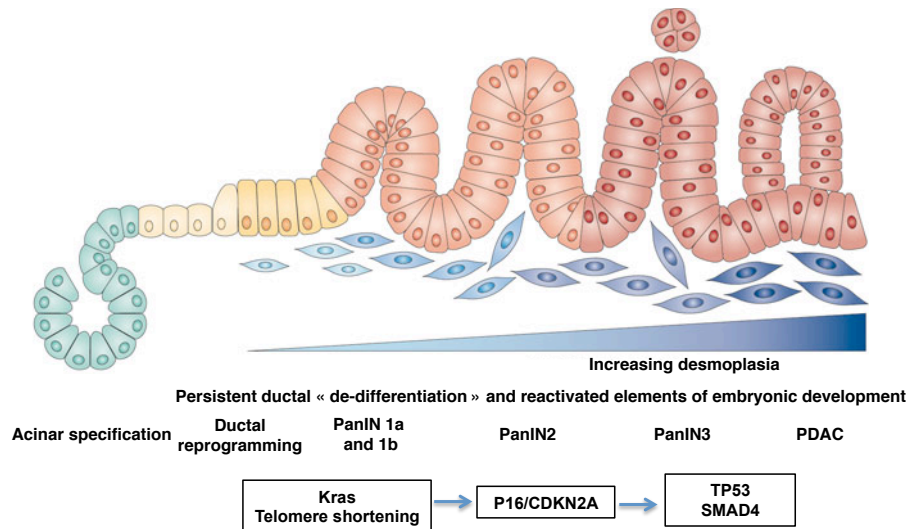


Figure 9. Model of PanIN progression (Adapted from Morris, et al., 2010). After pancreatic injury, acinar cells dedifferentiate into duct-like cells which can evolve to PDAC through PanIN lesions.

Genetic analyses of PanIN of different grades have shown the preponderance of mutations in the Kras gene (Kirsten rat sarcoma viral oncogene homolog). Kras is a member of a family that also comprises Harvey ras (Hras) and Neuroblastoma ras (Nras). They are GTPases that are part of the RAS/MAPK signaling pathway, and regulate cell growth, differentiation and apoptosis. GTPases function as a switch between “on” and “off” states, by binding to GTP and GDP molecules. The most frequent mutation of Kras in the pancreas occurs at codon 12, with substitution of a glycine to an aspartic acid residue. This mutation reduces the intrinsic ability of Kras to hydrolyse GTP, resulting in continuous stimulation of cell proliferation (Smit et al. 1988, Ellis and Clark 2000). Importantly, mutations in Kras appear as the earliest and most frequent genetic alteration in PanIN-1A (>99%) (Kanda et al. 2012).

Genetically engineered mouse models in which oncogenic Kras is expressed from its endogenous locus have demonstrated that oncogenic Kras

alone is not sufficient to induce PanIN and PDAC when activated at the adult stage, and that inflammation is required to initiate tumorigenesis (Guerra et al. 2007). A certain threshold of activated Kras seems to be critical for tumorigenesis. In line with this hypothesis, many studies have shown the presence of Kras mutations in healthy people suggesting the need of other factors to induce tumorigenesis (di Magliano and Logsdon 2013). It has been shown that caerulein injections lead to activation of NF- κ B; in the presence of oncogenic Kras, a positive feedback loop is initiated leading to sustained expression of Kras at a pathological level and development of PanIN (Daniluk et al. 2012). Kras has been demonstrated as a necessary driver of PanIN and PDAC formation, as inhibition of mutated Kras expression in developing neoplastic and cancerous lesions leads to their regression, even when one allele of the tumor suppressor p53 is lost (Collins et al. 2012). Interestingly, development of pancreatic neoplastic lesions driven by Kras is absolutely dependent on EGFR signaling (Ardito et al. 2012, Collins et al. 2012, Navas et al. 2012); although the mechanism is not well understood, it should be noted that Kras-driven tumorigenesis is EGFR-independent in lung and intestine (Navas et al. 2012).

In addition to activating Kras mutations, genetic analyses of PanIN have also shown telomere shortening as an early event in PanIN-1A development (van Heek et al. 2002). Another frequent mutation is the inactivating mutation in the tumor suppressor gene p16/CDKN2A. This mutation is found in 30% of PanIN-1, in 55% of PanIN-2 and 71% of PanIN-3 lesions (Wilentz et al. 1998). P16, also known as cyclin-dependent kinase inhibitor 2A, is implicated in cell-cycle regulation. The mutation leads to the loss of p16/CDKN2 protein expression and induces an acceleration of the cell cycle by facilitating the G1-S transition. Following the progression to high-grade PanIN lesions, other mutations are frequently found. Another commonly inactivated tumor-suppressor gene is the SMAD family member 4 (Smad4), mutated in approximately 30% of PanIN-3. SMAD4 is a key player in the signal transduction of TGF- β superfamily members from the cell surface to the nucleus (Wilentz et al. 2000). Finally, loss of TP53 occurs in the late carcinogenesis, with detection in late PanIN-3 grade. TP53 is a tumor suppressor

gene which controls cell-cycle regulation and apoptosis. Loss of p53 confers metastatic properties to oncogenic Kras-induced tumors in mouse models (Guerra et al. 2007, Koorstra et al. 2008). This loss also leads to independence of PDAC from EGFR signaling (Navas et al. 2012).

In summary, PanIN progressively accumulate many of the genetic alterations found in invasive PDAC and mutations of these genes are essential to support the evolution of PanIN into PDAC. Further improvements in our understanding of PanIN progression are needed to better prevent PDAC development (Figure 9).

d. Outline of PDAC development

Pancreatic ductal adenocarcinoma (PDAC), which accounts for 95% of cases of pancreatic cancer, (pancreatic neuroendocrine tumors account for less than 5%) is one of the most lethal forms of human cancer. It is the fourth leading cause of cancer death in the USA and could become the second leading cause of cancer-related death by 2030 (Rahib et al. 2014). The exceptionally poor prognosis is explained by the late detection due to the lack of diagnostic symptoms in the early stage of the disease and the limited response to current treatments. Only approximately 15% of patients diagnosed with PDAC undergo surgery. For others, chemotherapy and radiotherapy are usually proposed but the median survival of these patients is less than 6 months. Despite the improved understanding of pancreatic cancer biology, the overall five-year survival rate is approximately 5% and has remained unchanged over the last 30 years (Hezel et al. 2006, Eser et al. 2014). A lot of efforts have been devoted to understand the biology of this deadly cancer and a detailed view of the pathogenesis of PDAC is emerging; however how this cancer originates and how early detection can be achieved remain open questions.

As described above, genetic alterations have been characterized throughout the process of PDAC development. However factors at the origin of these genetic events are still unclear. With CP, the only well-established risk factor is cigarette smoking and 25% of pancreatic cancers could be attributed to smoking (Lowenfels and Maisonneuve 2006). Some demographic and lifestyle factors have also been associated with PDAC development, like age, male sex, black race, low socioeconomic status, alcohol consumption, diet, and obesity. People with adult-onset diabetes have also a 2-fold increased risk of pancreatic cancer (Yadav and Lowenfels 2013). A causal relationship between pancreatitis and PDAC has been demonstrated, putting forward a potential role for inflammation in pancreatic cancer development (Lowenfels et al. 1993).

Some genetic syndromes have also been associated with PDAC, and approximately 10% of pancreatic cancers are associated with inherited gene mutations (Algul et al. 2007). Several germline mutations have been identified which likely accelerate progression of premalignant to malignant lesions. For example, the Peutz-Jeghers syndrome (PJS) is caused by a mutation in a serine/threonine kinase 11/liver kinase B1 gene (STK11/LKB1); this syndrome is linked to the presence of polyps in the digestive tract and an increased risk to develop various kinds of cancers. Patients with this autosomal dominant disorder have a 132-fold increased risk of developing pancreatic cancer (Algul et al. 2007). Another autosomal dominant disorder linked to pancreatic cancer is the familial atypical mole and multiple melanoma (FAMMM) which is caused by mutations in the p16/CDKN2A gene. p16 mutation is associated with a 60%-90% melanoma risk by age 80 and an increase of 20% to develop pancreatic cancer by age 75 (Rustgi 2014). Also autosomal dominant germline mutation in the BRCA2 gene, which is famous for its increased risk for breast and ovarian cancers, increases risk for pancreatic cancer by 10-fold. BRCA2 plays role in DNA repair and is considered a tumour suppressor (Greer and Whitcomb 2007). Finally, the best recognized risk factor for PDAC is CP. In relation with this observation, germline mutations in the PRSS1 gene, which encodes a cationic trypsinogen protein, cause familial hereditary pancreatitis. Premature activation of the mutated trypsinogen

leads to autodigestion of acinar cells and exocrine insufficiency, as well as increased risk for PDAC. Lifetime risk for people with this type of mutations is increased 35-fold by the age of 70-75 (Rustgi 2014). This form of pancreatitis usually begins in late childhood leading to CP. This is the only known inherited syndrome for which the risk of cancer development is restricted to pancreatic cancer.

Currently, the best methods for surveillance have not been established for members from high-risk families; thus it is recommended that only centers experienced with inherited syndromes should follow these high-risk patients. Finally, a better understanding of mechanisms leading to ADM, PanIN and PDAC formation is urgently needed to identify the best targets to treat PDAC.

C- Primary Cilia

The involvement of cilia in the development of preneoplastic lesions constitutes a hypothesis in the present thesis. An outline of the role of this organelle in the pancreas is given in the following paragraphs.

a. Primary cilia organization

Cilia are evolutionarily conserved, antenna-like organelles that protrude from many cell types. Primary cilia are defined as non-motile, in opposition to motile cilia. They are unique in a cell. The only motile primary cilium is the nodal cilium, which moves to create nodal flow necessary for the establishment of morphogen gradients essential for left-right axis specification. Primary cilia are small ($<0.5\ \mu\text{m}$ in diameter) ranging from $1\ \mu\text{m}$ to $10\ \mu\text{m}$ in length. Until recently, they were thought to be a vestigial organelle but recent studies have demonstrated their role as sensor in many tissues, cilium disruption resulting in a broad spectrum of disorders (Michaud and Yoder 2006).

Primary cilia are usually displayed on differentiated quiescent cells. They are composed of a core, named axoneme, which is constituted of nine microtubule doublets, and a basal body (BB) located at the apical plasma membrane (Figure 10A). The BB is composed of a centrosome which acts as a microtubule-organizing center. The centrosome, surrounded by the pericentriolar matrix (PCM), is composed of two centrioles, the mother centriole and the daughter centriole. Centrioles are barrels of nine sets of microtubule triplets. When the cell reenters the cell cycle, the cilium and the basal body are disassembled. Centrioles are then free to function as the organizing center that ensures proper chromosome segregation. Each cell is composed of one older centriole (mother centriole), which acts as a template for the assembly of the daughter centriole during the division. Mother and daughter centrioles are morphologically distinct as mother centriole is accessorized by distal and sub-distal appendages (Figure 10B). Distal appendages are crucial for the recruitment of basal body to the membrane during cilia assembly

and sub-distal appendages seem to allow the anchoring of microtubules. They might be implicated in the regulation of cargo entry and exit of the cilium and mark the border of the plasma membrane and the ciliary membrane. They also play a role in the “ciliary pore complex” with the transition zone to regulate protein trafficking (Seeley and Nachury 2010, Kobayashi and Dynlacht 2011, Tanos et al. 2013). Centrioles are embedded in PCM which is a matrix of protein complexes. PCM serves as a platform for microtubule nucleation and anchors microtubules near the microtubule-organizing center.

Located between the BB and the axoneme, the transition zone is known to control ciliary protein composition by regulating intracellular trafficking. Even if the cilium membrane is continuous with the plasma membrane, the entry and exit of proteins into the cilium is tightly controlled and does not occur by simple diffusion. Proteins are delivered to the tip of the cilium by a process called Intraflagellar Transport (IFT). This bidirectional transport moves non-membrane bound particles along axonemal microtubules and is necessary for the assembly, maintenance and function of the cilium. IFT particles are composed of two subcomplexes, IFT-A (which seems to be linked to the retrograde transport) and IFT-B (for the anterograde transport). IFT-A and IFT-B dissociate after reaching the tip of the cilium and reassociate for the retrograde IFT. The BBSome, a complex of seven Bardet-Biedl syndrome (BBS) proteins, first associates the two subcomplexes at the ciliary base. It binds to the IFT complex for anterograde IFT, and is necessary for the correct recycling of these complexes into the cilium following dissociation of IFT-A and B complexes at the ciliary tip (Wei et al. 2012). Finally, the movement of the IFT complexes along the axoneme is mechanically achieved by two microtubule-based motor-proteins, kinesin-2 and dynein-2. Kinesin-2 (composed of KIF3A, KIF3B and KAP3) is implicated in anterograde transport while dynein-2 drives the retrograde transport.

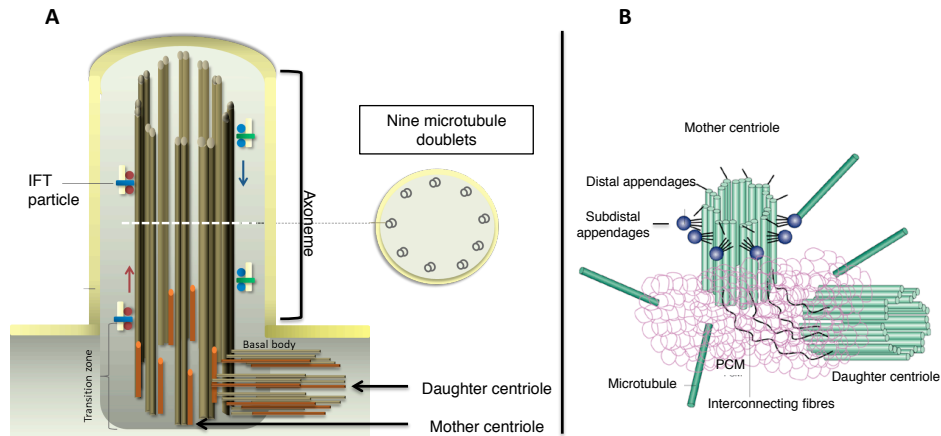


Figure 10. Ciliary structure (Adapted from Ainsworth, 2007 and Doxsey, 2001). **(A)** Primary cilium is composed of an axoneme constituted of nine microtubule doublets, and a basal body (BB). Proteins are delivered in the cilium by bidirectional intraflagellar transport (IFT). See text for additional details. **(B)** BB is composed of two centrioles linked by interconnecting fibres. Mother centriole has two sets of extra appendages, the distal and subdistal appendages. Centrioles are embedded in the pericentriolar matrix (PCM).

b. Functions of primary cilia

Over the last decade accumulating evidences have shown the biological importance of the primary cilium. The list of disorders categorized as ciliopathies has shed light on its role as sensor and cell-signaling hub. Morphologically, the cilium protrudes into the extracellular environment and can further transduce signals from extracellular stimuli to a cellular response. Thus the cilium can act as a mechanosensor. It has been shown that fluid flow induces cilium bending which results in increased intracellular Ca^{++} levels and subsequent modifications of multiple downstream processes. Most knowledge on mechanosensory mechanisms comes from studies of renal tubules. Patients with Autosomal Dominant Polycystic Kidney Disease (ADPKD) have mutations in the polycystin genes PC1 and PC2, which heterodimerize into an ion-channel complex. In

ADPKD, Ca^{++} entry into the cilium is decreased leading to defective flow sensing and formation of fluid-filled cysts and kidney failure (Nauli et al. 2003, Basten and Giles 2013). So, fluid flow sensation seems to be important to ensure tissue homeostasis.

Primary cilia also play roles as chemosensors. Indeed it was demonstrated that Hh signaling was largely dependent on cilium integrity. Currently, it is well known that in the absence of Sonic hedgehog (Shh) ligand, the Hh ligand binding receptor Patched (Ptc) localizes to the cilium whereas Smoothed (Smo), a G-protein coupled receptor is excluded from the cilium. When Shh binds to its receptor, Ptc is excluded from the cilium and relocalized at the plasma membrane, whereas Smo becomes activated and begins to accumulate at the ciliary membrane. Simultaneously, repression of transcription factors Gli2 and Gli3 by Suppressor of Fused Homolog (SuFu) is alleviated; this allows Gli2 and Gli3 stabilization and export to the nucleus where they drive the expression of target genes (Figure 11A). When primary cilium is not functional, the Hh signaling pathway is disrupted, resulting in multiple cell defects (Basten and Giles 2013).

Wnt signaling also seems to be associated with primary cilium. Knock-down experiments of ciliary-associated genes or some BBS genes result in hyperactive canonical Wnt response and increased levels of β -catenin (Gerdes et al. 2009, May-Simera and Kelley 2012). Cilium seems to support non-canonical signaling instead of canonical signaling (Figure 11B). One possible link between Wnt and primary cilium is the presence of the planar cell polarity component Inversin (Inv) which localizes at the base of the cilium. In presence of fluid flow, Ca^{++} entry in the cilium leads to Inv activation. Inv can then target cytoplasmic phosphoprotein dishevelled (Dsh) which results in its degradation via the Adenomatous Polyposis Coli and further inhibition of the canonical pathway. When cilium is absent or non functional, fluid flow is not monitored, leading to binding of Wnt ligands on frizzled receptor, to recruitment of Dsh, to subsequent inhibition of GSK-3 β complex, and to translocation of β -catenin in the nucleus (Figure 11B) (Berbari et al. 2009, May-Simera and Kelley 2012). Further studies will be needed to know the exact role of the cilium/BB in the regulation of this pathway even if

mutations affecting the function of the primary cilium usually lead to defects in the Wnt signaling (May-Simera and Kelley 2012).

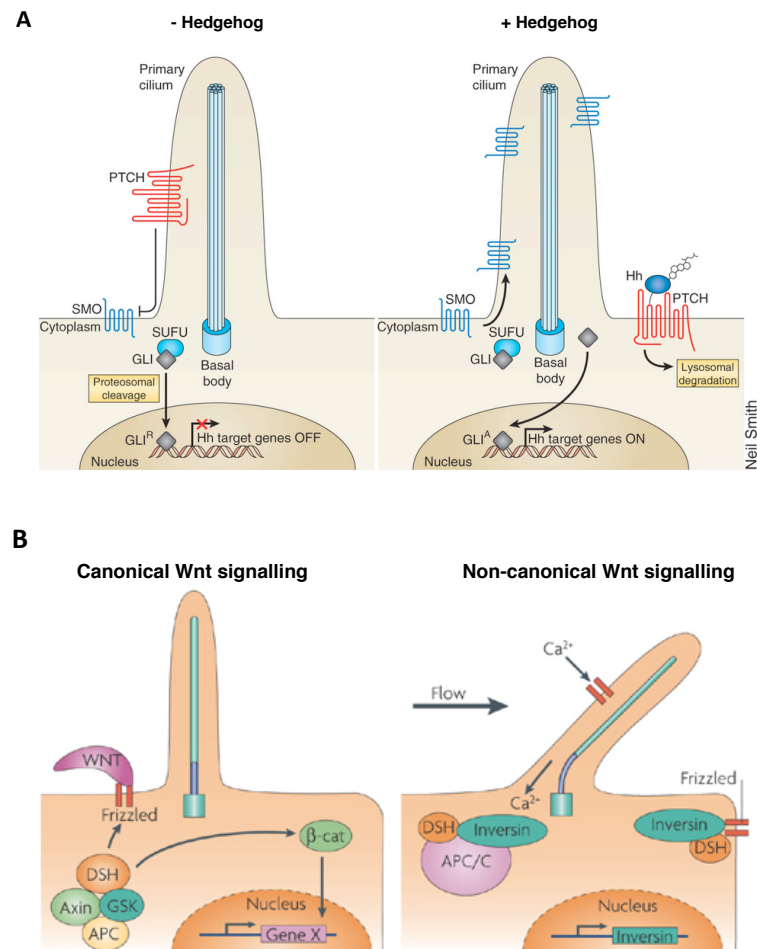


Figure 11. Hedgehog and Wnt signaling in cilia. (From Amakye et al., 2013 and Fliegeauf et al., 2007) **(A)** Left panel. In the absence of Hh ligand, the receptor Ptc is localized in the cilium and Smo is excluded from the cilia. Gli is processed to generate a transcriptional repressor. When Hh ligand binds to Ptc, Smo is relocalized at the ciliary tip; with ciliary exclusion of Ptc. Increased Smo levels are associated with activation of Gli and their entry to the nucleus to induce gene expression. **(B)** Fluid flow causes increased concentration of cytosolic Ca⁺⁺ and increases Inv expression. Inv targets cytoplasmic Dsh, making it unavailable for canonical Wnt signaling.

c. Primary cilia and pancreas

In pancreatic tissue, cilia are found in ductal and endocrine cells whereas they are absent from acinar cells. This difference could be explained by the high specialization of this cell type, with an important capacity of protein secretion. Presence of a cilium at the acinar apical pole has been hypothesised to interfere with the secretory machinery. As islet cells are also known for their capacity to secrete hormones, it has been pointed out that the apical surface of the acinar cells is smaller, which potentially does not allow separated sites for exocytosis and for the primary cilium (Seeley and Nachury 2010).

The role of primary cilia in the pancreas has remained unexplored for a long time but a connection between cilium abnormalities and pancreatic pathology has shed light on its importance for homeostasis of this organ. Ten percent of patients with polycystic diseases have pancreatic lesions, prompting investigation of the role of primary cilia in the pancreas (Malka et al. 1998). Several mouse models with mutations in IFT genes display pancreatic abnormalities. One of the best-characterized IFT mutations relates to the IFT88 gene which is required for cilium formation. Mutant mice develop a complex pancreatic phenotype with cystic lesions, acinar loss, fibrosis, ADM and alteration in the β -catenin expression (Cano et al. 2004, Zhang et al. 2005). In another mouse model, the gene coding for the kinesin-2 complex subunit, Kif3A, has been deleted. Specific deletion of Kif3A in the embryonic pancreas leads to a similar phenotype with cysts, acinar loss, fibrosis, ADM and lipomatosis (Cano et al. 2006). Interestingly, defects found in these mice are similar to those seen in CP.

In all these mouse models, deletion of cilia occurs during embryonic development and affects all epithelial cells of the pancreas. The specific function of primary cilia in pancreatic duct cells remains unexplored. Inactivation of the Kif3A gene in acinar and islet cells does not generate any obvious phenotype, suggesting a role of ductal cilia in the phenotype observed in the Kif3A KO mice (Cano et al. 2006). These models also raise the intriguing question whether the acinar cell phenotype is caused by ductal defects or whether it originates from an

early and cilium-independent role of the ciliary protein during pancreas development. Another interesting observation is the development of ADM lesions in mice with defective primary cilia (Cano et al. 2006). ADM lesions can progress to neoplastic lesions and further PDAC. Currently no causal link between primary cilia dysfunction and PDAC has been demonstrated, even if PanIN lesions and PDAC cells are devoid of primary cilia.

D- MiRNAS

a. Biogenesis

MicroRNAs (miRNAs) are small non-coding RNAs of ~22 nucleotides which postranscriptionally regulate gene expression. Currently more than ~ 3000 miRNAs have been found in humans (Friedlander et al. 2014). Most of them are highly conserved, suggesting important biological functions. Indeed they participate in the regulation of almost all biological processes demonstrating crucial roles for cell and tissue homeostasis (Bartel 2004). MiRNAs are located in intergenic regions but they are also found in introns or exons from coding genes. Frequently their expression is controlled by a specific promoter but alternatively, they share the same promoter with their associated gene (Cai et al. 2004, Lin et al. 2006).

In mammalian cells, miRNAs are transcribed by RNA polymerase II into primary miRNAs which are capped and polyadenylated, in a similar way to conventional mRNAs. The pri-miRNA is then processed by a complex of proteins containing the RNase III enzyme, Drosha. After cleavage by this microprocessor complex, the precursor miRNA (pre-miR), which has a double-stranded (ds) stem-loop structure, is exported from the nucleus to the cytoplasm by Exportin-5, a Ran-GTP dependent dsRNA-binding protein. Finally a second RNase III, Dicer, cleaves the pre-miRNA to produce a ~22-nucleotide duplex. One miRNA strand is incorporated into the RNA Induced Silencing Complex (RISC) of which contains Dicer, TRBP and the Argonaute protein Ago2, and induces postranscriptional gene silencing by interacting with target genes (Figure 12).

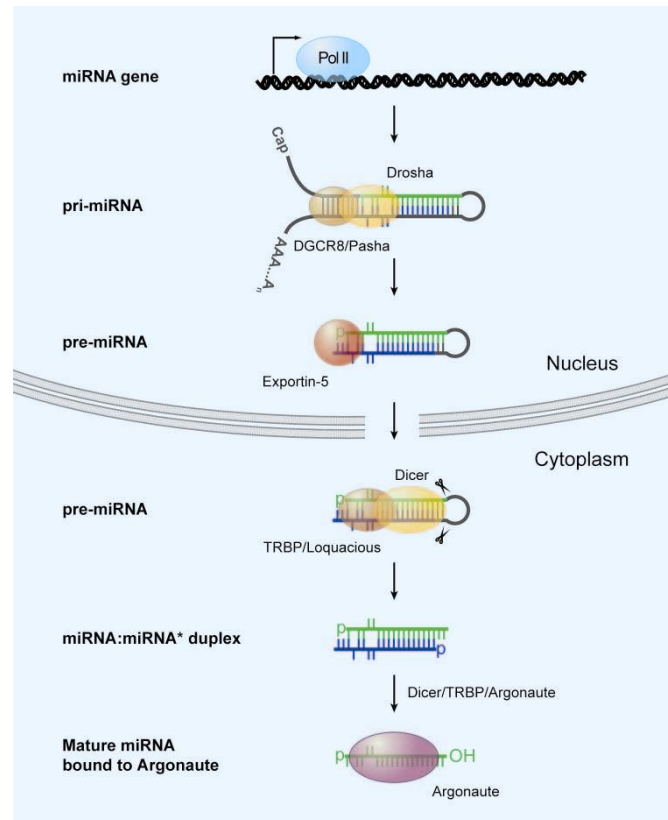


Figure 12. MiRNAs biogenesis. See text for additional details. (Bushati and Cohen 2007)

b. MiRs and cancer

MiRNAs are involved in essential biological processes and their deregulation is linked to diseases like cancer. Depending on their targets, miRNAs act either as tumor suppressors, or as oncogenes, called oncomiRs (Jansson and Lund 2012). OncomiRs are involved in the regulation of genes supporting tumor growth, invasion and angiogenesis (Hayes et al. 2014).

MiRNAs are frequently downregulated in cancer, due to epigenetic modifications or defects during their biogenesis (Lu et al. 2005). This was firstly

characterized for miR-15 and miR-16 in B-cell chronic lymphocytic leukemias (Calin et al. 2002). Since then, numerous studies have shown aberrant expression of miRNAs during cancer progression. Studying modification of miRNAs in cancer has contributed to identify mechanisms of tumor formation and to classify cancer types (Rosenfeld et al. 2008). MiRNAs are also used as prognosis indicators. One of the best studied oncomiR is miR-21, which is virtually up-regulated in all solid tumors (Krichevsky and Gabriely 2009). In a mouse model of lung cancer, it has been demonstrated that inhibition of miR-21 partially protected against tumor formation (Hatley et al. 2010). Thus, understanding which miRNAs are specifically involved in tumor development could help to develop treatments to inhibit or restore expression of these non-coding RNAs. Currently one clinical trial aims to restore the expression of miR-34a which is downregulated in a subset of solid tumors (Hayes et al. 2014). Moreover, an increasing number of studies are trying to correlate miRNA signature with tumor formation in order to use them as non-invasive biomarkers.

V. AIMS OF THE WORK

Pancreatic ductal adenocarcinoma is one of the deadliest cancers. The exceptionally poor prognosis of this cancer is explained by the late detection which results from the lack of diagnostic symptoms at the early stages of the disease. Understanding the mechanisms leading to development of PDAC is required to improve early detection and treatment. Recent studies have demonstrated that PDAC can originate from acinar cells. When acinar cells are exposed to inflammation, they undergo acinar-to-ductal metaplasia, which consists in the transdifferentiation of acinar cells into duct-like cells. These metaplastic lesions are considered pre-cancerous lesions because they can progress to neoplastic lesions (PanIN) and further to invasive cancer.

The overarching goal of my thesis is to characterize mechanisms leading to preneoplastic transformation of acinar cells. My host lab has previously demonstrated the ectopic presence of two transcription factors, namely Hnf6 and Sox9, in human metaplastic acinar cells. It has also shown that Hnf6 is required to induce ADM in acinar cells. Therefore, my objectives were to determine how expression of Hnf6 is controlled in pancreatic acinar cells and how Hnf6 expression in ductal cells affects development of ADM. I also planned to determine how Sox9 expression in acinar cells promotes development of neoplastic lesions.

The first objective of my thesis was to test the hypothesis that Hnf6 expression is controlled by miRNAs in normal differentiating acinar cells. My work contributed to the identification of gene network involving miRNAs and transcription factors whose perturbation induces acinar metaplasia. This study constitutes the first part of the Results section.

In the second part of my thesis I collaborated with a PhD student in my laboratory to implement an *in-vitro* assay to study metaplasia formation in the presence or absence of Sox9. In this study, we identified an oncogenic role of Sox9 in pancreatic cancer, which is exerted by activation of the EGFR pathway.

Finally, in the last part of my thesis I focused on the role of Hnf6 in the maintenance of adult ductal cell fate. It was previously shown that conditional deletion of this transcription factor during embryogenesis led to metaplastic lesions, suggesting that ductal expression of Hnf6 in adult ducts is required for pancreatic homeostasis. To test this hypothesis I specifically deleted Hnf6 in adult pancreatic ducts and found that loss of ductal cell homeostasis leads to ADM. These results are compiled in my main scientific paper which was recently submitted for publication.

VI. RESULTS

A- Let-7b and miR-495 Stimulate Differentiation and Prevent Metaplasia of Pancreatic Acinar Cells by Repressing HNF6

Article published in Gastroenterology 2013 Sep; 145 (3): 668-678.e3. Published Online First: 16 May 2013

In the first part of my thesis, we addressed the role of miRNAs in acinar cell differentiation. We performed gain- and loss-of functions experiments, both in *in vitro* and *in vivo* settings, using two mouse models deficient for miRNAs. In this work, I characterized the phenotype of the Ela-Cre/Dicer^{loxP/-} to understand the role of miRNAs in acinar cells. I also helped to obtain, analyze and interpret data of Figures 4, 5, 6.

Abstract

BACKGROUND & AIMS:

Diseases of the exocrine pancreas are often associated with perturbed differentiation of acinar cells. MicroRNAs (miRNAs) regulate pancreas development, yet little is known about their contribution to acinar cell differentiation. We aimed to identify miRNAs that promote and control the maintenance of acinar differentiation.

METHODS:

We studied mice with pancreas- or acinar-specific inactivation of Dicer (Foxa3-Cre/Dicer(loxp/-) mice), combined (or not) with inactivation of hepatocyte nuclear factor (HNF) 6 (Foxa3-Cre/Dicer(loxp/-)/Hnf6-/- mice). The role of specific miRNAs in acinar differentiation was investigated by transfecting cultured cells with miRNA mimics or inhibitors. Pancreatitis-induced metaplasia was investigated in mice after administration of cerulein.

RESULTS:

Inhibition of miRNA synthesis in acini by inactivation of Dicer and pancreatitis-induced metaplasia were associated with repression of acinar differentiation and with induction of HNF6 and hepatic genes. The phenotype of Dicer-deficient acini depends on the induction of HNF6; overexpression of this factor in developing acinar cells is sufficient to repress acinar differentiation and to induce hepatic genes. Let-7b and miR-495 repress HNF6 and are expressed in developing acini. Their expression is inhibited in Dicer-deficient acini, as well as in pancreatitis-induced metaplasia. In addition, inhibiting let-7b and miR-495 in acinar cells results in similar effects to those found in Dicer-deficient acini and metaplastic cells, namely induction of HNF6 and hepatic genes and repression of acinar differentiation.

CONCLUSIONS:

Let-7b, miR-495, and their targets constitute a gene network that is required to establish and maintain pancreatic acinar cell differentiation. Additional studies of this network will increase our understanding of pancreatic diseases.

B- SOX9 regulates ERBB signalling in pancreatic cancer development

Article published in Gut 2015 Nov; 64 (11): 1790-1799. Published Online First: 21 October 2014

In the second chapter of the Results section, I collaborated with a PhD student of the host laboratory to study the role of Sox9 in PanIN formation. Specifically, I designed and set-up an *in vitro* assay using mouse primary pancreatic cells to study the formation of metaplastic complexes in the presence or in the absence of Sox9, in a Kras-dependent context. I performed the experiments presented in Figure 5, and participated to the analysis and interpretation of the results, and to the drafting of this figure. I also contributed to finalize the paper by performing several experiments requested by the reviewers.

Abstract

OBJECTIVE:

The transcription factor SOX9 was recently shown to stimulate ductal gene expression in pancreatic acinar-to-ductal metaplasia and to accelerate development of premalignant lesions preceding pancreatic ductal adenocarcinoma (PDAC). Here, we investigate how SOX9 operates in pancreatic tumourigenesis.

DESIGN:

We analysed genomic and transcriptomic data from surgically resected PDAC and extended the expression analysis to xenografts from PDAC samples and to PDAC cell lines. SOX9 expression was manipulated in human cell lines and mouse models developing PDAC.

RESULTS:

We found genetic aberrations in the SOX9 gene in about 15% of patient tumours. Most PDAC samples strongly express SOX9 protein, and SOX9 levels are higher in classical PDAC. This tumour subtype is associated with better patient outcome, and cell lines of this subtype respond to therapy targeting epidermal growth factor receptor (EGFR/ERBB1) signalling, a pathway essential for pancreatic tumourigenesis. In human PDAC, high expression of SOX9 correlates with expression of genes belonging to the ERBB pathway. In particular, ERBB2 expression in PDAC cell lines is stimulated by SOX9. Inactivating Sox9 expression in mice confirmed its role in PDAC initiation; it demonstrated that Sox9 stimulates expression of several members of the ERBB pathway and is required for ERBB signalling activity.

CONCLUSIONS:

By integrating data from patient samples and mouse models, we found that SOX9 regulates the ERBB pathway throughout pancreatic tumourigenesis. Our work opens perspectives for therapy targeting tumourigenic mechanisms.

C- Chronic pancreatitis and lipomatosis are associated with defective function of ciliary genes in pancreatic ductal cells

In this chapter, we addressed the role of Hnf6 in adult ductal cells using mice with a deficiency of this factor in their ductal cells. Their phenotypic characterization showed that they developed pancreatitis and lipomatosis. This was very similar to the phenotype of mice with a ductal deletion of the kinase Lkb1; the latter mice were analyzed in the laboratory by Louis Collet. In both mouse models, we observed defects in the primary cilia of the mutant ductal cells associated with pancreatitis. Therefore, we merged the results from both analyses in a single paper. I contributed to study concept and design, acquisition of data, analysis and interpretation of data and writing the manuscript. Louis Collet performed all experiments on Lkb1-deficient mice.

**Chronic pancreatitis and lipomatosis are associated with
defective function of ciliary genes in pancreatic ductal cells**

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Abstract

Genetic diseases associated with defects in primary cilia are classified as ciliopathies. Pancreatic lesions and ductal cysts are found in patients with ciliopathic polycystic kidney diseases suggesting a close connection between pancreatic defects and primary cilia. Here we investigate the role of two genes whose deletion is known to cause primary cilium defects, namely *Hnf6* and *Lkb1*, in pancreatic ductal homeostasis. We find that mice with postnatal duct-specific deletion of *Hnf6* or *Lkb1* show duct dilations. Cells lining dilated ducts present shorter cilia with swollen tips, suggesting defective intraciliary transport. This is associated with signs of chronic pancreatitis, namely acinar-to-ductal metaplasia, acinar proliferation and apoptosis, presence of inflammatory infiltrates, fibrosis and lipomatosis. Lipomatosis results in part from transdifferentiation of acinar cells. Our data reveal a tight association between ductal ciliary defects and pancreatitis with perturbed acinar homeostasis and differentiation. Such injuries can account for the increased risk to develop pancreatic cancer in Peutz-Jeghers patients who carry *LKB1* loss-of-function mutations.

Introduction

Cilia are evolutionarily conserved, antenna-like organelles that protrude from cells into the extracellular environment and transduce signals from extracellular stimuli to the recipient cells. Over the last decade accumulating evidences have shown the biological importance of the primary cilium. Disruption of cilium structure or function is responsible for an increasing number of disorders categorized as ciliopathies (1,2). These diseases have shed light on the role of cilia as sensors and signalling hubs (3-6).

Like in other organs, ciliary defects are associated with dysfunctions of the pancreas. In this organ, exocrine functions are exerted by the acinar cells, which secrete digestive enzymes, and by the ductal cells lining the ducts, which transport and modify acinar secretions to the duodenum. Within the pancreas, ductal and islet cells are ciliated whereas acinar cells are not. A link between ciliary defects and pancreatic abnormalities was initially suggested by the observation that patients with autosomal dominant polycystic kidney disease (ADPKD; OMIM entry #173900), a ciliopathy, have pancreatic cysts and develop chronic pancreatitis (7, 8). Subsequently mouse models revealed that deletion of ciliary genes leads to cystic ducts, acinar-to-ductal metaplasia, acinar apoptosis, and lipomatosis (9-11).

In the studies listed above, disruption of the ciliary genes, cyst formation and ciliary defects were initiated during the foetal period. In addition, this disruption abolished gene functions in all pancreatic cell types, leaving open the question of the cell type whose defect is causing the diseases. Since cilia are found in duct cells and not acinar cells, specific ductal ablation of genes involved in ciliary function is essential to fully understand the role of cilia in the pancreas (11).

Transcription factor Hepatocyte Nuclear Factor 6 (Hnf6/Onecut1) is involved in key aspects of pancreas development (12-14), including in pancreatic duct development: constitutive inactivation of Hnf6 leads to pancreatic cyst formation characterized by the absence of primary cilia during embryogenesis (15). Likewise, the tumour suppressor Liver Kinase B1/Serine/Threonine Kinase 11 (Lkb1/Stk11) has also been involved in development of pancreatic cysts (16). No link has currently been established between pancreatic deletion of Lkb1, the

presence of these cysts and ciliary defects. However, such a link can be hypothesised since *Lkb1* is present in the primary cilium (17) and *Lkb1* loss results in ciliary defects (18).

To investigate the role of *Hnf6* and *Lkb1* in ductal cells and their relation with primary cilia, we postnatally inactivated the two genes specifically in pancreatic ductal cells. *Hnf6*- and *Lkb1*-deficient mice displayed very similar phenotypes characterized by enlarged ducts, acinar apoptosis, acinar-to-ductal metaplasia, and lipomatosis which resulted in part from transdifferentiation of acinar cells. Importantly, both *Hnf6*- and *Lkb1*-deleted mice showed reduced length and swelling of primary cilia found in the dilated ducts, indicative of perturbed ciliary function. Our data underscore the tight association between ciliary defect and pancreatitis, suggesting that perturbed homeostasis and ciliary function of the ductal cells can cause pancreatitis with perturbed differentiation of acinar cells.

Results

Postnatal ductal deletion of Hnf6 or Lkb1 induces lipomatosis

To study the phenotypic consequences of postnatal Hnf6 loss in ductal cells, we generated mice that harbor floxed Hnf6 alleles and carry a Sox9-CreER transgene which allows tamoxifen-dependent and duct-specific recombination of target genes (Sox9CreER/Hnf6^{fl/fl} mice). Nursing mothers were treated with tamoxifen to induce Hnf6 deletion in their offspring at postnatal days 2 to 10 by transfer of tamoxifen via milk (Figure 1A). Hnf6 deletion was observed in about 75% of the ductal cells (Supplementary Figure 1).

Three weeks after birth, examination of Sox9CreER/Hnf6^{fl/-} pancreata revealed slight pancreatic abnormalities. Ductal lumens were dilated and a few infiltrating adipocytes were observed near dilated ducts (Figure 1B). At this stage, acinar cell morphology appeared unaffected (Figure 1B). With age, an increasing number of adipocytes were detected in the Sox9CreER/Hnf6^{fl/-} pancreata. This culminated in 6-month old animals with a subset of acinar lobules being entirely replaced by adipocytes (Figure 1B).

Concomitantly, we characterized the pancreatic phenotype of mice with ductal deletion of Lkb1 (Sox9CreER/Lkb1^{fl/fl} mice). Lkb1 deletion was achieved postnatally (Figure 1A), as described for Hnf6 inactivation. The lack of Lkb1 antibody suitable for detection of Lkb1 on PFA-fixed paraffin sections precluded determination of efficiency with which Lkb1 was deleted in Sox9CreER/Lkb1^{fl/fl} ducts. However the pancreatic phenotype exhibited by Sox9CreER/Lkb1^{fl/fl} mice (see below) suggests that this deletion was efficient. Lipomatosis was detected like in Sox9CreER/Hnf6^{fl/-} pancreas. The phenotypic consequences of Lkb1 deletion were delayed as compared to those in the absence of Hnf6, since this phenotype appears beyond 3 weeks of age (Figure 1B-C). Despite extensive replacement of acinar cells by adipocytes in both mouse models, no body wasting was observed, suggesting that the remaining acini were sufficient to ensure homeostasis (data not shown).

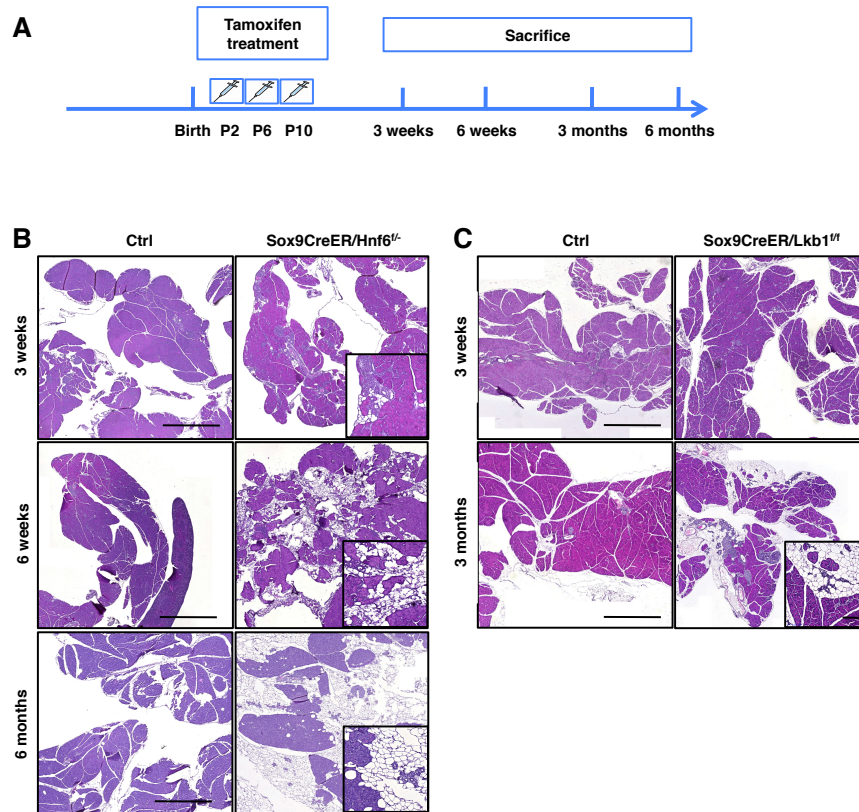


Figure 1. Histological analysis shows similar fatty infiltration in Sox9CreER/Hnf6^{fl/-} and Sox9CreER/Lkb1^{fl/fl}. (A) Schedule of Hnf6 and Lkb1 deletion. Nursing mothers were injected with tamoxifen by intraperitoneal injections at postnatal days (P) 2, 6 and 10. Hematoxylin and eosin (H&E) staining of pancreatic tissues from control (Ctrl) and mutant mice. (B) Three-week old Sox9CreER/Hnf6^{fl/-} mice show only few adipocytes with dilated ducts. At 6 weeks and 6 months, a large part of the acinar tissue is replaced by adipocytes. (C) Three-week old Sox9CreER/Lkb1^{fl/fl} mice show no phenotype, while at 3 months of age, dilated ducts and adipocytes are found in the pancreatic tissue. Scale Bar= 1 mm.

The origin of adipocytes infiltrating the pancreas was addressed by lineage tracing. Since previous studies showed that acinar cells can transdifferentiate into adipocytes (19, 20), we labelled and traced the acinar cells in Sox9CreER/Hnf6^{fl/-} pancreas using a doxycycline-controlled transgenic system (Figure 2). The latter consists of an elastase promoter-driven tetracycline transactivator (tTA) expressed in acinar cells, which in the absence of doxycycline, binds to a TetO element located upstream of a flippase (Flp)-coding gene. Flp then recombines Flp recognition target (FRT) sites flanking a stop cassette (FSF) located upstream of an YFP gene inserted in the Rosa locus (Rosa FSF YFP mice), leading to YFP expression in the acinar cells (Figure 2A). In control mice, adipocytes labelled by Fabp4 were found at the periphery of the pancreas but none of these cells were YFP-positive (data not shown). In contrast, a few adipocytes were positive for YFP in Sox9CreER/Hnf6^{fl/-} pancreas, suggesting that adipocytes derived at least in part from acinar cells in these mice (Figure 2B). Expression of β -catenin and its target c-Myc was reduced in Sox9CreER/Hnf6^{fl/-} acinar cells, suggesting that defective expression of these genes contributes to acinar-to-adipocyte transdifferentiation, as observed by others in Myc-deficient acinar cells (Bonafant et al. 2009) (Figure 2C). Finally, genetic lineage tracing of the ductal cells in Sox9CreER/Hnf6^{fl/-}/RosaYFP pancreas did not provide evidence for duct-to-adipocyte transdifferentiation (Figure 2D).

Together our results indicate that ductal deletion of Hnf6 and Lkb1 induces similar phenotypes, albeit with different timing of events. Extensive lipomatosis develops, resulting in part from acinar-to-adipocyte transdifferentiation.

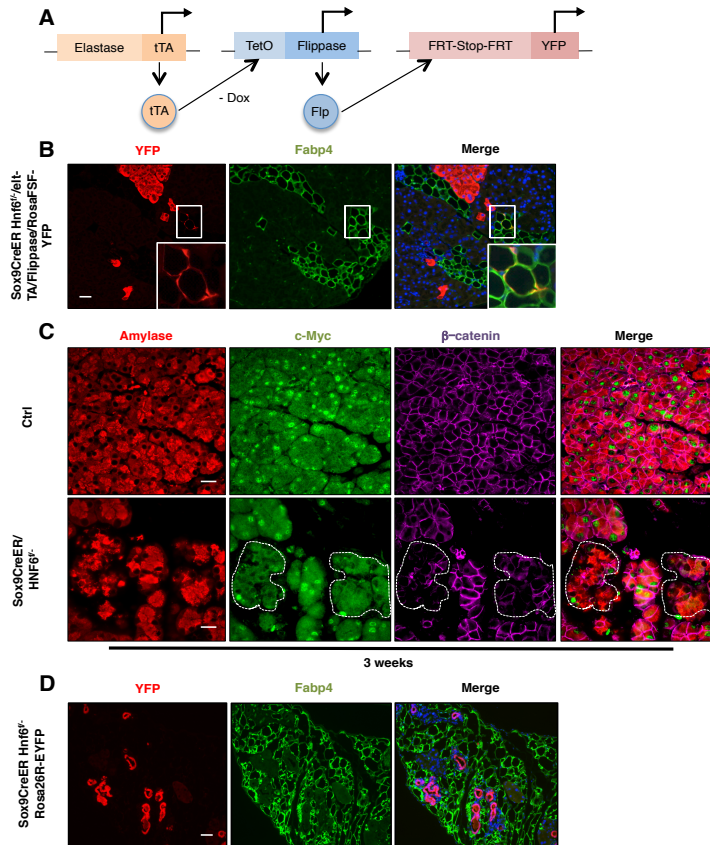


Figure 2. Acinar cells, but not ductal cells, can transdifferentiate into adipocytes. (A) Schematic structure of the constructs used for acinar lineage tracing. (B) In Sox9CreER HNF6^{fl/-}/elt-TA/Flippase/RosaFSF-YFP mice, few Fabp4-positive adipocytes are stained with YFP, suggesting that acinar cells transdifferentiate into adipocytes. DAPI was used in the merge picture to label the cell nuclei. Scale bar= 50 μm. (C) In Sox9CreER HNF6^{fl/-} pancreas, some metaplastic acinar cells (surrounded by dashed lines) show a decreased expression of c-myc and β-catenin, compared to control pancreas. Scale bar= 50 μm. (D) In Sox9CreER HNF6^{fl/-}/Rosa26R-EYFP mice, ductal lineage tracing shows no colocalization between the ductal marker YFP and the adipocyte marker Fabp4. DAPI was used in the merge picture to label cell nuclei. Scale bar= 50 μm.

Fibrosis and inflammation are detected in Sox9CreER/Hnf6^{fl/-} and Sox9CreER/Lkb1^{fl/fl} pancreata

Masson's trichrome staining showed periductal fibrosis in mice with ductal deletion of Hnf6 and Lkb1 (Figure 3A). Fibrotic regions were positively stained for the intermediate filament protein vimentin, a marker frequently up-regulated in pancreatitis (Figure 3B). In control pancreata, vimentin expression was only observed around large pancreatic vessels and ducts (Figure 3B).

As fibrosis and inflammation are often associated during pancreatitis, we also looked for the presence of inflammatory infiltrates and detected the presence of macrophages throughout pancreata of Sox9CreER/Hnf6^{fl/-} and Sox9CreER/Lkb1^{fl/fl} mice (Figure 3C). Macrophages were absent from control pancreata (Figure 3C).

We concluded that inactivation of Hnf6 and Lkb1 leads to lipomatosis associated with chronic pancreatitis.

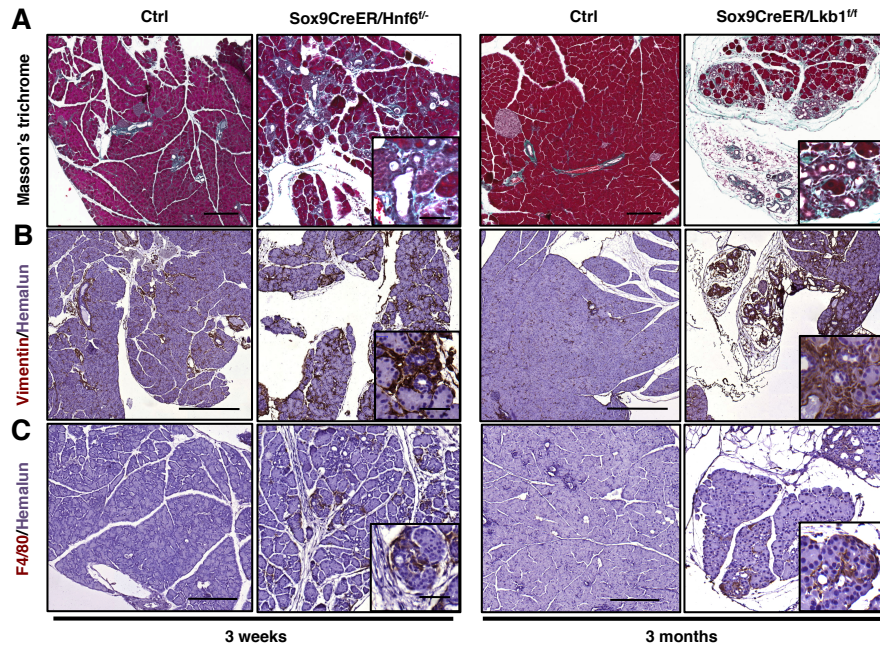


Figure 3. Phenotypic analysis of *Sox9CreER/Hnf6^{fl/-}* and *Sox9CreER/Lkb1^{fl/fl}* pancreata. (A) Masson's trichrome staining (blue-green) reveals significant increase in collagen deposition in periductal regions in *Sox9CreER/Hnf6^{fl/-}* and *Sox9CreER/Lkb1^{fl/fl}* pancreas. Scale bar= 200 μ m. (B) Staining for the intermediate filament vimentin confirms the presence of fibrosis in *Sox9CreER/Hnf6^{fl/-}* and *Sox9CreER/Lkb1^{fl/fl}* pancreata. Scale bar= 500 μ m. (C) F4/80 staining shows the presence of macrophages in *Sox9CreER/Hnf6^{fl/-}* and *Sox9CreER/Lkb1^{fl/fl}* mice. Scale bar= 200 μ m, inset= 50 μ m

Pancreatic ducts are dilated in Sox9CreER/Hnf6^{fl/-} and Sox9CreER/Lkb1^{fl/fl} pancreata

Dilated pancreatic ducts were frequently observed in the fibrotic parts of Sox9CreER/Hnf6^{fl/-} and Sox9CreER/Lkb1^{fl/fl} pancreata (Figure 3B-D). Sox9 labelling and quantification of the duct area revealed that the average duct lumen exhibited an 8- and 5-fold increase in Sox9CreER/HNF6^{fl/-} and Sox9CreER/Lkb1^{fl/fl} ducts, respectively, as compared to controls (Figure 4A). No significant increase in ductal cell proliferation was observed in 3- and 6-week old pancreata in the absence of Hnf6 (Figure 4B). In contrast, hyperplastic areas of Sox9CreER/Lkb1^{fl/fl} pancreata, showed increased ductal proliferation (Figure 4B). This suggests that in these animals, increased ductal proliferation causes ductal dilatation.

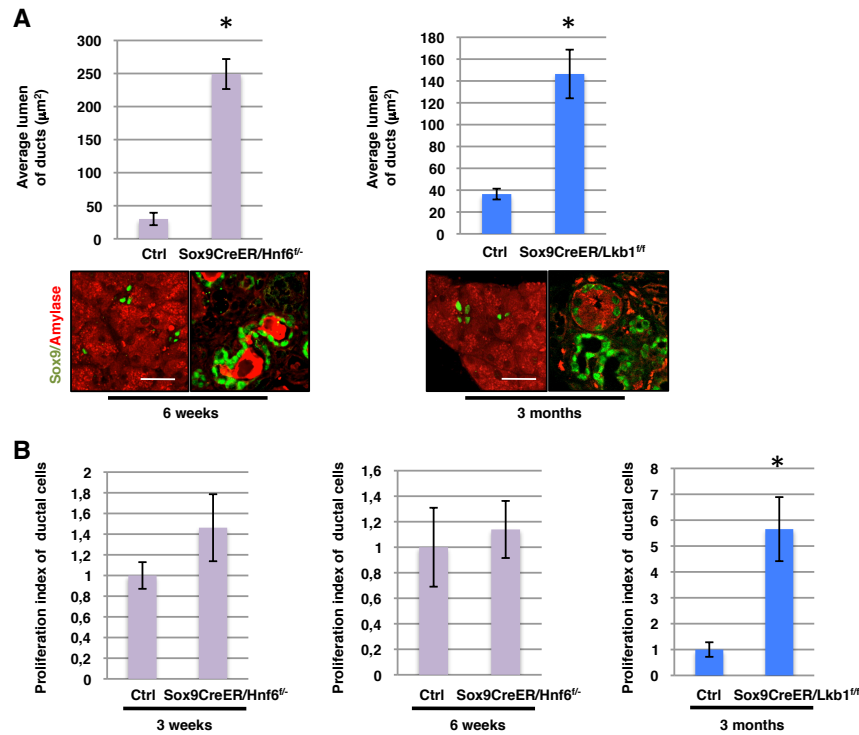


Figure 4. Pancreatic ducts are dilated in Sox9CreER/Hnf6^{f/-} and Sox9CreER/Lkb1^{f/f} pancreata. (A) Amylase and Sox9 labelling shows that ductal inactivation of Hnf6 significantly increases the area of the pancreatic duct lumen by 8.3-fold (29.9 ± 9.4 vs 249.2 ± 22.7 , $*P < 0.05$). An identical phenotype is observed in Lkb1-deficient ducts with an increase of 4.0-fold (36.4 ± 4.9 vs 146.4 ± 22.3 , $*P < 0.05$). Scale bar= 20 μm (B) Proliferation index of ductal cells, calculated as the percentage of Sox9-positive cells that are Ki67-positive cells, was determined in control (ctrl), Sox9CreER/Hnf6^{f/-} and Sox9CreER/Lkb1^{f/f} pancreata. No significant difference was found between control and Hnf6-deficient pancreata at 3 weeks or 6 weeks after birth (control, $n=4$; Hnf6-deficient pancreas, $n=5$). Three months after birth, Lkb1-deficient ductal cells showed a 5.6-fold increased proliferation (1.0 ± 0.3 vs 5.8 ± 1.3 , $*P < 0.05$), as compared to control mice (control, $n=3$, LKB1-deficient pancreas, $n=3$). Values are means $\pm$ sem.

Ductal deletion of Hnf6 or Lkb1 causes acinar-to-ductal metaplasia, proliferation, and apoptosis

Pancreatic inflammation and fibrosis are often associated with acinar-to-ductal metaplasia (ADM), a process characterized by a down-regulation of acinar genes and up-regulation of ductal genes in the metaplastic acinar cells (21). Immunofluorescent labelling for the acinar markers *Mist1*, *Ptf1a* and amylase showed decreased or lack of expression in a subset of acinar cells of the *Sox9CreER/Hnf6^{fl/-}* and *Sox9CreER/Lkb1^{fl/fl}* pancreata (Figure 5A-B).

Consistent with ADM, the transcription factors *Hnf6* and *Sox9* showed ectopic expression in metaplastic acinar cells, and were detected exclusively in ductal cells in control mice (Figure 5C and D). Likewise, we also detected induction of *ErbB2*, a member of the Epidermal Growth Factor Receptor (EGFR) pathway known to be induced in metaplastic cells (22, 23) (Figure 5E).

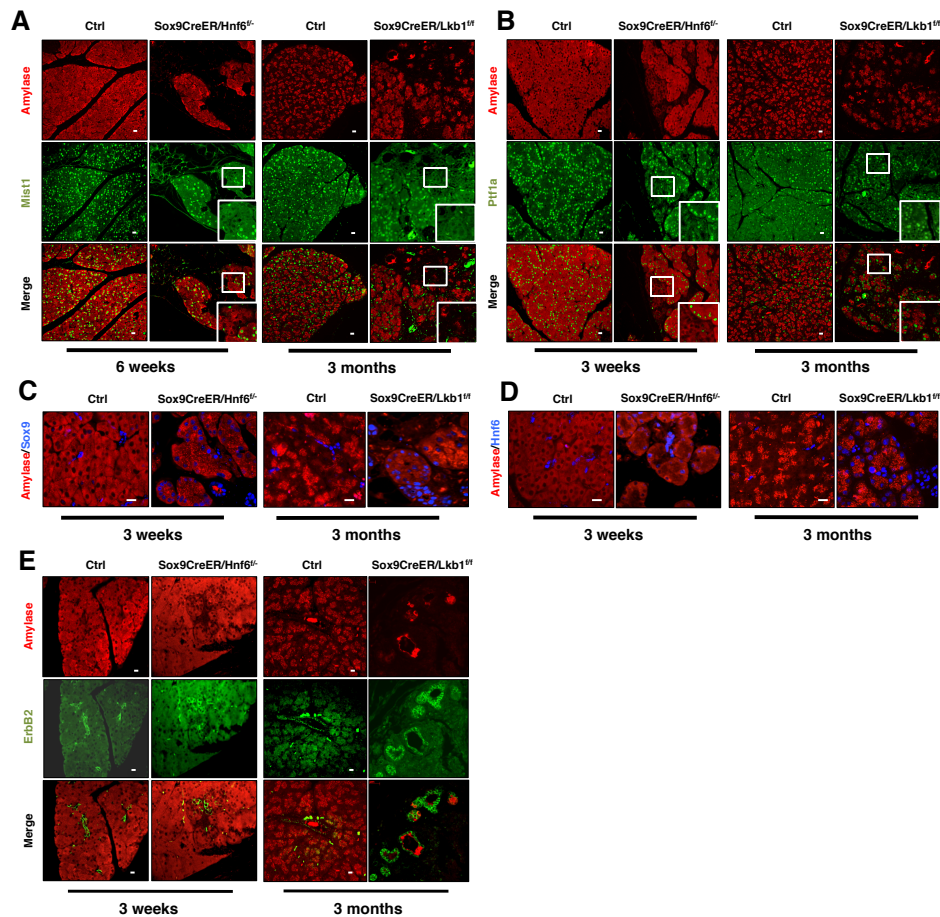


Figure 5. Ductal deletion of *Hnf6* or *Lkb1* leads to acinar-to-ductal metaplasia.

(A, B) Immunofluorescence detection of the acinar markers *Mist1*, *Ptf1a*, and *amylase*. Acinar markers are decreased in a subset of lobules (insets). (C) Immunofluorescence labelling of the ductal transcription factor *Sox9* reveals extensive staining in acinar cells of *Sox9CreER/Hnf6^{-/-}* or *Lkb1^{fl/fl}* mice compared to control mice in which *Sox9* expression is restricted to ductal cells. (D) Expression of *Hnf6* is high in ducts and absent in acinar cells in control pancreas. In *Sox9CreER/Hnf6^{-/-}* or *Lkb1^{fl/fl}* pancreas, expression of *Hnf6* is increased in metaplastic acinar cells. (E) The EGFR signalling pathway member *ErbB2* is ectopically induced in acinar cells of *Sox9CreER/Hnf6^{-/-}* pancreata compared to control tissue. Scale bar= 50 μm.

Proliferation of acinar cells in mutant pancreas was assessed by immunolabelling of Ki67. In Sox9CreER/Hnf6^{f/-} pancreata, significantly increased proliferation was detected starting at 6-weeks of age (Figure 6A). Average proliferation was increased 3-fold in Sox9CreER/Lkb1^{f/f} pancreata at 3 months. However, due to high variability between metaplastic areas in Lkb1-deficient pancreata, the latter results were not statistically significant (Figure 6C). Metaplastic areas in mutant Sox9CreER/Hnf6^{f/-} and Sox9CreER/Lkb1^{f/f} pancreata also showed increased numbers of apoptotic acinar cells, as detected by TUNEL staining (Figure 6B-D).

Together, these data suggest that ductal deletion of Hnf6 or Lkb1 induces pancreatitis associated with acinar-to-ductal metaplasia, apoptosis and proliferation of acinar cells.

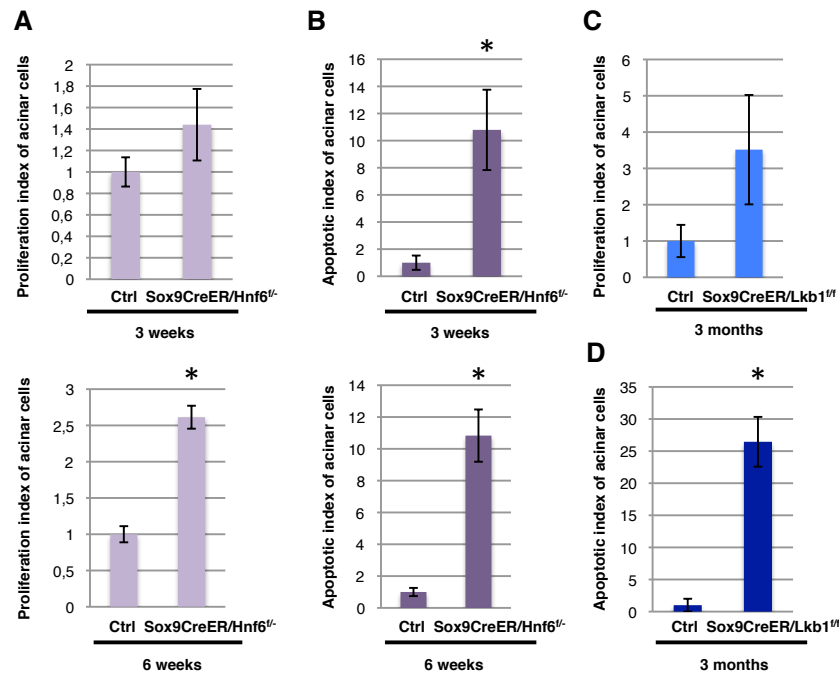


Figure 6. Acinar proliferation and apoptosis are increased in mutant pancreata. (A) Proliferation indexes of acinar cells are given as the percentage of *Ptf1a*-positive cells expressing Ki67 (3-week old animals, control, $n=5$, *Hnf6* mutant, $n=5$; 6-week old animals, control, $n=5$, *Hnf6* mutant, $n=3$). Acinar proliferation was increased in *Hnf6*-deficient ductal cells at 3 weeks, by 1.5-fold (8.1 ± 1.1 vs 11.7 ± 2.7) but did not reach statistical significance. At 6 weeks, proliferation was significantly increased by 2.5-fold (2.7 ± 0.3 vs 7.0 ± 0.4 , $*P < 0.05$). (B) Quantification of apoptotic cells was measured as the number of cells positive for TUNEL staining per pancreas field (control, $n=3$, *Hnf6* mutant, $n=3$). TUNEL staining showed increased apoptosis in mutant mice at 3 weeks by 10.9-fold (0.06 ± 0.03 vs 0.6 ± 0.2) and 6 weeks by 10.8-fold (0.1 ± 0.03 vs 1.4 ± 0.2 , $*P < 0.05$). (C) *Sox9CreER/Lkb1^{fl/fl}* mice showed an increased though not statistically significant proliferation of acinar cells (control, $n=3$, *Lkb1*, $n=3$). (D) TUNEL staining showed increased apoptosis in mutant mice at 3 months by 26.4-fold (0.02 ± 0.02 vs 0.6 ± 0.08 , $*P < 0.05$). Values are means $\pm$ sem.

Defective cilia are found in Hnf6- and Lkb1-deficient ductal cells

Similarities between mice lacking Hnf6 or Lkb1 in their pancreatic ductal cells suggest that both phenotypes depend on overlapping mechanisms. Since deletion of these genes in embryonic cells results in loss of primary cilia (15, 18) we analysed cilia in pancreatic ducts cells of the mutant mice. We used antibodies against acetylated tubulin, a component of the cilium axoneme, and against Arl13b, a small GTPase that localizes to cilia and regulates ciliary length and signalling (24). Cilia were present in both control and mutant mice but were reduced in length by 2.5-fold in Sox9CreER/Hnf6^{fl/-} and by 1.6-fold in Sox9CreER/Lkb1^{fl/fl} ductal cells (Figure 7A and C). Swelling of the ciliary tip was also detected (Figure 7A-B), and this was confirmed by Z-stacking analysis (Supplementary Figure 2).

Previous reports have shown that ciliary defects, duct dilatation and apical cell polarity defects are sometimes associated (18, 25). However, we found no evidence for abnormal apical polarity of the mutant ductal cells since ezrin and Mucin1A were normally located at the apical pole of the ductal cells (Supplementary Figure 3A-B). Therefore, the absence of Hnf6 or Lkb1 affects specifically the primary cilium and is not the result of global apico-basal perturbation.

Taken together, these data indicate that Hnf6 or Lkb1 deletion in pancreatic ductal cells results in ciliary defects.

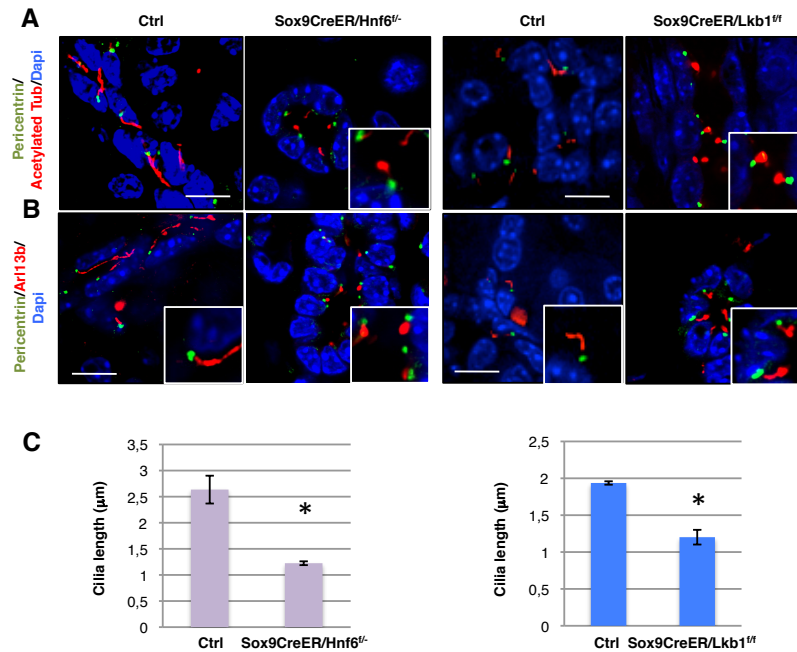


Figure 7. Primary cilia are shorter and show a swollen tip in mutant ductal cells. (A) Cilium axoneme was visualized using an antibody against acetylated tubulin. Centrioles located at the basal bodies were labelled by pericentrin. DAPI labels the ductal cell nuclei. Insets reveal the swelling of the ciliary tip in Hnf6- and Lkb1-deficient cells. (B) Labelling by Arl13b confirms cilium swelling in the mutant cells (insets). Scale bar= 10 μm. (C) Cilium length is reduced in both mutant pancreata. Hnf6 mutant cells show a decrease in the cilium length by 2.5-fold (2.6 ± 0.3 vs 1.2 ± 0.04 , control, $n=3$; Hnf6, $n=3$) whereas a 1.6-fold decrease (1.9 ± 0.02 vs 1.2 ± 0.1) is observed in Lkb1 mutant cells. $*P < 0,05$. Values are means $\pm$ sem.

Discussion

Over the last years a growing number of ciliopathies have been discovered. However little is known about the role of primary cilia in the pancreas. Development of ductal pancreatic cysts and pancreatitis in ciliopathies like polycystic kidney diseases suggested a role of ciliary dysfunction in pancreas disease. Pancreatic deletion of the genes coding for Kinesin Family Member 3a (Kif3a) and Intraflagellar Transport 88 (Ift88), which are required for cilium formation and maintenance, further supported this hypothesis (9-11). However these studies were conducted with mouse strains expressing Cre recombinase in the pancreatic progenitors. This prevented to draw conclusions about the cell type whose defects cause the pancreatic anomalies. In this paper, we show that duct-specific deletion of Hnf6 or Lkb1, two genes with functions previously linked to the cilia, leads to ciliary defects, lipomatosis, and pancreatitis. Considering the phenotypic similarities between mouse models deficient in Hnf6, Lkb1, Kif3a, and Ift88 (9-11) our data strongly suggest that ciliary defects contribute to pancreatitis and lipomatosis.

Hnf6 is required for duct morphogenesis in the developing pancreas. Constitutive deletion of Hnf6 leads to cystic ducts and defects in assembly of primary cilia (15). Interestingly, no sign of ADM and pancreatitis is observed in the HNF6 KO mice (12). Unlike in Sox9CreER/Hnf6^{fl/-} mice, Hnf6 cannot be induced in acini of constitutive Hnf6 KO mice. Since induction of Hnf6 in acini is necessary for ADM, constitutive Hnf6 KO mice cannot develop ADM (21). In contrast ductal deletion of Hnf6 is associated with acinar expression of this factor, thereby allowing ADM.

Lkb1 is found in primary cilia (26) and its loss in cultured fibroblasts results in disassembly of the cilium (18). In pancreas, Lkb1 deletion at the embryonic stages leads to pancreatitis and cystic neoplasms (16). This observation is consistent with a strongly increased risk of pancreatic cancer in Peutz-Jeghers syndrome patients (PJ; OMIM entry #175200) who carry germline inactivating mutations of Lkb1 (27). The present work is to our knowledge the first showing ciliary defects resulting from Lkb1 inactivation in an animal model. We suggest that

pancreatic cancer risk in PJ patients and ciliary defects are linked. Whether ciliary defects directly promote cancerogenesis, or whether cancer indirectly results from chronic pancreatitis remains to be determined. Interestingly, three genes with ciliary functions, *ExoC4*, *Ift80*, and *Dock1*, were identified as potentially involved in familial pancreatic cancer (28, 29). Moreover, ADPKD patients sometimes develop intraductal papillary mucinous tumour (30), a neoplasm arising from pancreatic ductal cells.

Duct dilations in our mouse models may result from duct obstruction and such obstruction may cause pancreatitis. We do not favour this hypothesis. First, duct obstruction is expected to generate an acute response, while our phenotypes develop slowly and gradually increase over several weeks. Second, duct obstruction in mice, as seen after pancreatic duct ligation, generates a massive inflammatory response with little or no lipomatosis (21). Instead, discrete inflammatory infiltrates and very significant lipomatosis are monitored in the absence of ductal expression of *Lkb1* or *Hnf6*.

Unlike in *Kif3a*-deficient pancreata (11), *Hnf6*- and *Lkb1*-deficient duct cells are not devoid of cilia but show short cilia with a swollen tip: ciliary proteins, like acetylated tubulin and *Arl13b*, accumulated at the ciliary tips. Similar ciliary morphology is found when expression of retrograde IFT proteins, such as *Dync2h1*, *Rp2*, *Ift122*, and a subset of Bardet-Biedl syndrome proteins (31-33) is affected. This suggests that in the absence of *Hnf6* or *Lkb1*, retrograde IFT is defective with accumulation of proteins at the apex of the cilium.

In conclusion, our results support a model in which perturbed ductal homeostasis resulting from ciliary defects leads to lipomatosis and acinar injuries. Therefore, we suggest that a ciliopathy may be a cause of idiopathic pancreatitis and eventually contribute to cancerogenesis.

Materials and Methods

Mice

Hnf6^{+/-}, Hnf6^{ff}, Lkb1^{ff}, Sox9-CreERT, Rosa LSL YFP, Elastase-tTA, Rosa FSF YFP mice have been described elsewhere (12, 34-39). Mice received humane care according to the criteria listed by the Belgian National Academy of Sciences. All strains were maintained in an enriched CD1 background.

Tamoxifen treatment

Nursing mothers were treated with an intraperitoneal injection of 100 μ L tamoxifen (Sigma-Aldrich, Saint-Louis, Missouri, USA) dissolved in corn oil (Sigma) at a concentration of 30 mg/ml combined with the gavage of another 100 μ L on days 2, 6 and 10 after birth.

Immunohistochemistry and immunofluorescence

Pancreata were fixed for 6 hours in 4% paraformaldehyde at 4°C prior to paraffin embedding. Serial sections (7 μ m) were analysed. Immunofluorescence protocol was performed as in Prévot *et al* (40). Primary antibodies are listed in Supplementary Table 1. The anti-amylase antibody batch used to analyse sections from Sox9CreER/Hnf6^{ff} mice differs from the one used for analysis of Sox9Cre/Lkb1^{ff} sections, resulting in slightly different staining results. Masson's trichrome staining was achieved by deparaffinising and rehydrating the tissue, then placing the sections in Hematoxylin solution for 1 min. Slides were rinsed and placed into fuchsin-xylidine-ponceau stain for 5 min. After additional rinsing, slides were placed into 1% phosphomolybdic acid solution for 5 min and then transferred into 1% light green solution (L1886, Sigma-Aldrich) for 3 min. Slides were then rinsed with acidified water and dehydrated through a series of anhydrous alcohol before mounting. Fluorescence labelling was visualized and photographed with a Cell Observer Spinning Disk confocal microscope (Zeiss, Zaventem, Belgium). A Mirax imaging system (Zeiss) was used after histological staining. Rabbit anti-Sox9 antibodies were raised against a peptide located between amino acids 470 and

520 of the human Sox9 protein. No cross-reactivity with Sox10 was found.

Hyperplastic area quantification

Following immunolabelling for Sox9 and Amylase, 12 non-overlapping, representative tissue fields at 25X magnification were randomly chosen to measure area of ductal lumina. For transgenic mice, only hyperplastic areas were used for quantification. Only ducts composed of more than 3 cells were taken into account. Axiovision software was used to measure the internal area of ducts.

Proliferation and apoptosis

Proliferation of duct and acinar cells was determined by immunolabeling with Ki67 and Sox9 or Ptf1a antibodies. Positive cells were scored from 7 non-overlapping fields at 25X magnification. The percentage of Ki67 positive cells was calculated by dividing the number of cells expressing Ki67 by the total number of cells expressing Sox9 or Ptf1a. For ductal proliferation, hyperplastic areas from transgenic mice were used for counting. The level of apoptosis was determined using the transferase-mediated dUTP nick end-labelling (TUNEL, Roche Diagnostics Co.) assay according to the manufacturer's protocol. Apoptosis was quantified by counting the number of labelled nuclei per mouse (5 fields for each mouse). The percentage of TUNEL-positive cells was calculated by dividing the number of acinar cells positive expressing TUNEL staining by the total number of Ptf1a-positive acinar cells. Hyperplastic and metaplastic area were used for counting. All countings were performed with Adobe Photoshop CS5.

Quantification of cilia length

Immunofluorescent images of cilia stained for acetylated tubulin were taken using a Cell Observer Spinning Disk confocal microscope at 100X magnification (Zeiss, Zaventem, Belgium). Approximately 50 cilia were measured for each mouse (n=3 for each conditions). Data analysis was performed with Axiovision software.

Statistical Analysis

Statistical significance was determined using the non-parametric Mann-Whitney's U-test. Differences were considered significant for $P < 0.05$. Statistical analyses were carried out with GraphPad Prism 5.0 (GraphPad Software, Inc, La Jolla, CA, USA).

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Conflict of Interest Statement

The authors declare no competing interests.

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Authors Contributions

CA: study concept and design, acquisition of data, analysis and interpretation of data, statistical analysis, drafting and revision of manuscript. LC: study concept and design, acquisition of data, analysis and interpretation of data, statistical analysis, revision of the manuscript. CG, MB: material support, revision of the manuscript. FPL, PJ: study concept and design, analysis and interpretation of data, drafting and revision of manuscript, obtaining funding.

Ethics approval

All animal experiments were performed with the approval of the animal welfare committee of the University of Louvain Medical School.

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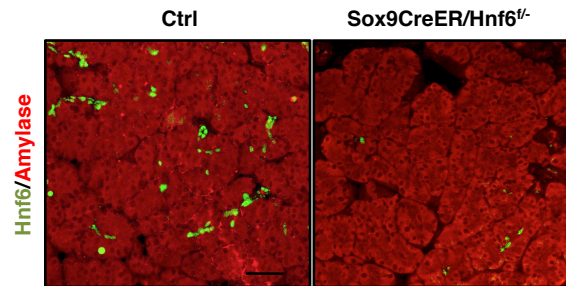
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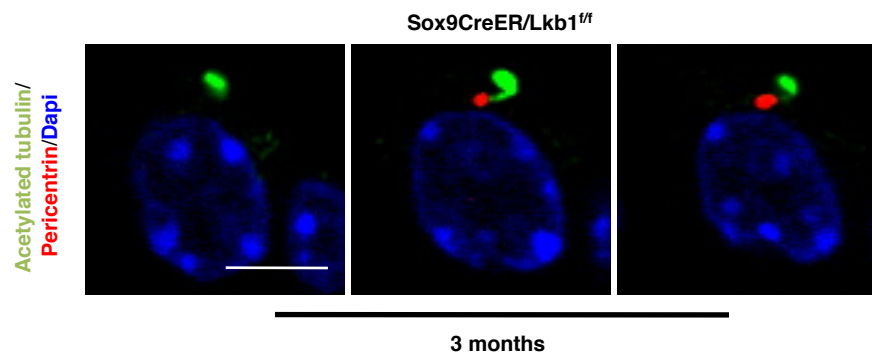
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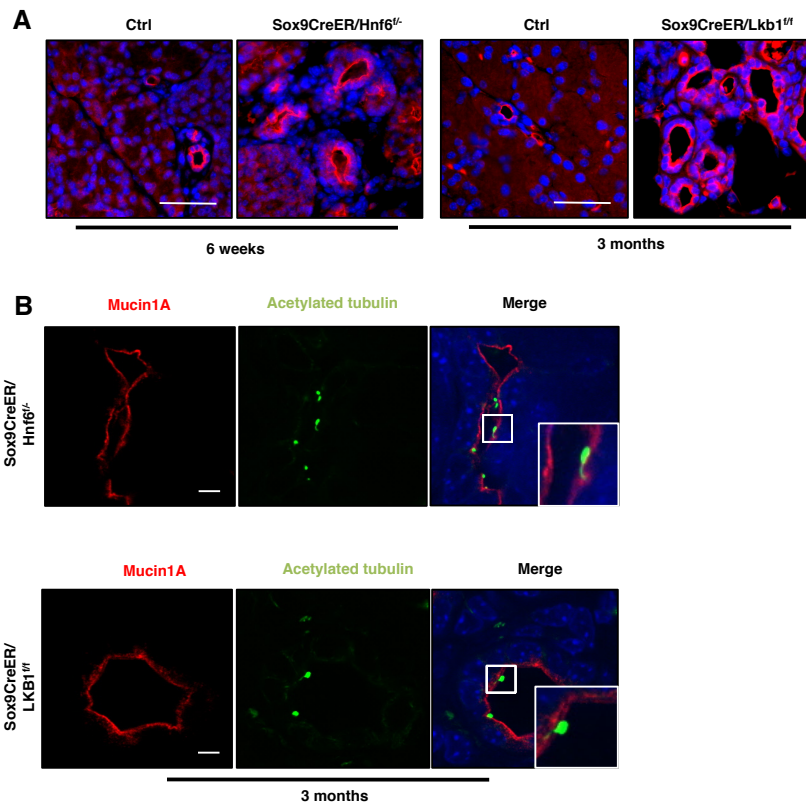
Supplementary Figures



Supplementary Figure 1. Ductal deletion of *Hnf6* at the postnatal stage. Immunofluorescence of *Hnf6* and *Amylase* performed on control (Ctrl) and *Sox9CreER/Hnf6^{fl/-}* mice. *HNF6* was deleted in about 75% of the ductal cells of *Sox9CreER/Hnf6^{fl/-}* pancreata. Scale Bar = 50 μ m.



Supplementary Figure 2. Z-stack series of a *Lkb1*-deficient primary cilium shows swelling of the ciliary tip. Acetylated tubulin labelling suggests accumulation of axonemal materials at the ciliary tip. DAPI labels the ductal cell nuclei. Scale bar= 5 μ m.



Supplementary Figure 3. Apical polarity of the mutant ductal cells is not perturbed. (A) Ezrin labelling is correctly localized in dilated ducts of Sox9CreER/Hnf6^{-/-} and Sox9CreER/Lkb1^{-/-} pancreata. Scale bar= 50 μ m. (B) Mucin1A labelling shows no loss of ductal polarity even if associated with reduced cilia length. Scale bar= 5 μ m.

Supplementary Table 1. Antibodies used for immunohistochemical analysis

Antibodie	Origin	Reference	Dilution
Acetylated tubulin	Mouse IgG2	Sigma (T6793)	1/2500
Amylase	Goat	Santa Cruz (sc-12821)	1/500
Amylase	Rabbit	Sigma (A8273)	1/500
Arl13b	Mouse IgG2	NeuroMab (73-287)	1/200
ErbB2	Rabbit	DAKO (A0485)	1/600
Ezrin	Mouse	NeoMarkers (MS-661-P1)	1/200
F4/80	Rat	Biolegend (122602)	1/500
Fabp4	Rabbit	Abcam (ab13979)	1/300
GFP	Goat	Abcam (ab6673)	1/250
HNF1b	Rabbit	Atlas antibody (HPA00208)	1/200
HNF6	Guinea Pig	Described in Prévot et al (Prevot et al. 2012)	1/10 000
Ki67	Mouse	BD (556003)	1/250
Mist1	Mouse	Santa Cruz (sc-80984)	1/200
Mucin1a	Armenian Hamster	NeoMarkers (HM-1630-P1)	1/1000
Pericentrin	Rabbit	Eurogentec (PRB-432C)	1/2000
Ptf1a	Rabbit	B. Bréant	1/500
Sox9	Rabbit	de Duve Institute	1/1000
Vimentin	Rabbit	Cell Signaling (D21H3)	1/200
Secondary Antibodies	Origin	Reference	Dilution
Anti-guinea pig	Goat	Molecular Probes (A11076)	1/1000
Alexa Fluor 594	Donkey	Jackson ImmunoResearch (706-605-148)	1/1000
Anti-goat	Donkey	Molecular Probes (A11078 / A21446)	1/1000
Alexa Fluor 488 / 647	Donkey	Molecular Probes (A11001 / A11005)	1/1000
Anti-mouse	Donkey	Molecular Probes (A11001 / A11005)	1/1000
Alexa Fluor 488 / 594	Donkey	Molecular Probes (A11008 / A11012)	1/1000
Anti-rabbit	Donkey	Molecular Probes (A11008 / A11012)	1/1000
Alexa Fluor 488 / 594	Donkey	Molecular Probes (A11008 / A11012)	1/1000
Biotinylated-anti-armenian hamster	Goat	Abcam (ab5744)	1/1000
HRP-anti-rabbit	Goat	Molecular Probes (T-20922)	1/100
Biotinylated-Anti-rat	Goat	Vector Laboratories (BA-9401)	1/1000
Anti-streptavidin-POD	-	Roche (1-089-153)	1/1000

VII. CONCLUSIONS, GENERAL DISCUSSION AND PERSPECTIVES

Pancreatic ductal adenocarcinoma is known for its extremely poor prognosis. Lack of symptoms and of diagnostic methods to detect early tumorigenic events explains the high mortality. Over the last two decades our understanding of this pathology has increased but without significant improvement in the survival rate of this cancer. It was previously thought that PDAC was initiated from ductal cells but accumulating evidences have demonstrated that PDAC arise from acinar cells (Kopp et al. 2012). Upon inflammation, acinar cells undergo dramatic changes in differentiation and acquire a ductal morphology, a phenomenon called ADM. ADM is considered a preneoplastic lesion, as it potentially derives into PanIN and further into invasive cancer. Therefore understanding and defining mechanisms initiating ADM is critical to improve treatments; these mechanistic questions were at the centre of my thesis.

In the first part of my work, we focused on the role of miRNAs in the initiation of ADM. To do so, we inactivated Dicer, an enzyme essential for miRNA maturation during pancreas development, and found that a network of transcription factors and miRNAs is necessary to promote acinar differentiation and maturation. We demonstrated that two miRNAs, let-7b and miR-495 target transcription factor Hnf6 to repress its expression in differentiating acinar cells and thus to prevent ADM.

In the second part of my thesis, I collaborated to a study on the role of Sox9 in pancreatic tumorigenesis. My host laboratory had previously identified a role for Sox9 in ADM (Prevot et al. 2012) but the function of Sox9 in PDAC initiation was unknown. During this work, we demonstrated that Sox9 plays a key role in the development of ADM and neoplastic lesions by regulating the activity of the ErbB pathway.

Finally, we initiated a study on the role of Hnf6 in adult pancreatic ductal cells. By deleting Hnf6 specifically in postnatal ductal cells, we uncovered a new role for this transcription factor in the maintenance of pancreatic homeostasis. We

observed that primary cilia are deficient in this model, and that ciliary defects are tightly associated with pancreatitis and perturbed homeostasis of the acinar cells.

A- Importance of miRNAs to prevent ADM formation during developmental and oncogenic processes

My host laboratory resorted to its expertise in pancreatic cell differentiation to unravel the mechanisms of ADM. It demonstrated the importance of ductal transcription factors in this process but how these transcription factors are regulated during normal and perturbed cell differentiation is unknown. MiRNAs are small RNAs which participate to the accurate regulation of biological processes (Sayed and Abdellatif 2011). These molecules play major roles in embryonic development and disease, and several studies have shown modifications in miRNA expression during pancreatitis and PDAC (Bloomston et al. 2007). To study if such perturbations already arise at the metaplastic stages, we performed a microarray experiment on metaplastic versus control acinar cells. We found modifications in the expression of 39 miRNAs; most displayed increased expression in metaplastic acinar cells as compared to normal acinar cells (data not shown); experiments are in progress to characterize the role of the highest induced microRNA in ADM and PanIN development. In parallel to this study, we also tested the possibility that miRNAs are important to allow correct pancreatic differentiation. The role of miRNAs during pancreatic development had been previously studied but most of the studies were focused on their role during endocrine differentiation (Lynn et al. 2007, Dumortier and Van Obberghen 2012). We demonstrated that miRNAs play a role during acinar differentiation: precise levels of let-7b and miR-495 are necessary to repress Hnf6 and allow acquisition of the acinar phenotype, as miRNA deficiency leads to acinar metaplasia (Prevot et al. 2013). Another step in our understanding of the role of miRNAs in regulation of acinar homeostasis was added a year ago by a study in which Dicer was specifically deleted in adult acinar cells (Wang et al. 2014). Here also, acinar cells underwent metaplastic conversion and when mutated Kras was expressed in these miRNA-deficient acinar cells, neoplastic lesions developed within the pancreatic tissue. An interesting observation mentioned in this study is the sensitivity to variation in Dicer concentration. Indeed, when heterozygous deletion of Dicer was performed, the

number of PanIN lesions was much higher compared to WT mice. However, in homozygous Dicer KO mice, formation of metaplastic structures was accelerated but metaplasia never progressed to PanIN. Authors conclude that the presence of miRNAs at an appropriate level is required for tumor progression (Morris et al. 2014, Wang et al. 2014). Thus miRNAs are important players for the maintenance of acinar cell identity both in the embryonic and adult pancreas. Our work and that of others reveal not only that microRNAs are key determinants of cell identity in homeostatic condition and disease, but also highlight that they exert their function within a well-defined range of concentration.

B- Distinct roles of Hnf6 and Sox9 during preneoplastic and neoplastic development

Since Hnf6 plays a key role during development of pancreatic ducts and since ectopic induction of Hnf6 in developing acinar cells activates ductal genes, we also studied its role in inflammation-induced ADM in adult pancreas.

The laboratory has previously shown that Hnf6 and Sox9 are up-regulated in human and murine metaplastic cells. Consequently, we focused on the role of these two transcription factors during ADM. To address this question, the laboratory first used a mouse model of pancreatitis induced by pancreatic duct ligation (PDL). In this model, the main pancreatic duct is ligated which ultimately leads to inflammation, ADM and apoptosis of most acinar cells. After ligation, similarly to what happened during embryonic development when let-7b and miR-495 were repressed, metaplastic cells showed up-regulation of Hnf6, Sox9 and of ductal genes, and down-regulation of acinar genes. When PDL was performed on mice deleted for Hnf6, ADM was blocked. Deletion of Sox9 inhibited but did not abrogate ADM, indicating that inactivation of Sox9 is less protective against metaplasia than Hnf6 deletion; this demonstrated a primordial role for these two transcription factors in ADM. Moreover, ectopic expression of Hnf6, but not Sox9,

was sufficient and necessary to induce ADM (Prevot et al. 2012). In conclusion, Hnf6 is required for converting the acinar phenotype into a ductal phenotype both during embryogenesis and in adult tissue.

We also studied the role of Hnf6 and Sox9 in the subsequent stage of the tumorigenic process, namely, the formation of neoplastic lesions. To do so, we induced inflammation by injections of caerulein. We decided to select this method as PanIN had been shown to develop in mice expressing an oncogenic form of Kras following caerulein-induced pancreatitis; in contrast, no study had validated the PDL model to obtain PanIN.

Using the caerulein model, we observed that mice lacking Sox9 in acini developed less ADM than control mice. More importantly, these mice were completely protected against caerulein-induced PanIN development in a Kras dependent-context, demonstrating an oncogenic role of Sox9 during tumorigenesis. This indicates that Sox9 is required for the initiation and the development of PanIN driven by oncogenic Kras in caerulein-induced inflammation (Kopp et al. 2012, Grimont et al. 2015).

We also performed the same experiments on mice lacking Hnf6 in acini, in the presence or absence, of oncogenic Kras. In both cases, no protective effect of the absence of Hnf6 on ADM and PanIN development was seen in the pancreas (data not shown). The discrepancy with the results obtained in the PDL model is likely explained by activation of different signaling pathways and divergent inflammatory responses, during duct-ligation or caerulein-induced pancreatitis. While molecular mechanisms operating in caerulein-induced inflammation have been extensively characterized (see Figure 5 of the introduction), PDL-induced inflammation has not been investigated. Consequently molecular mechanisms of PDL and caerulein-induced inflammation have never been systematically compared.

Interestingly, it was previously demonstrated that injection of a cholecystokinin-receptor antagonist during PDL led to reduced hyperamylasemia without reduction of oedema. Therefore, it is then possible that cholecystokinin (a caerulein analog) mediates some effects of duct ligation (Ohshio et al. 1991). Indeed, when the main

pancreatic duct is ligated, this initiates a decrease in pancreatic juice secretion in the enteral lumen which induces an upregulation of enzyme production by acinar cells via a neurohormonal response (Meyerholz and Samuel 2007). During PDL, ligation of the main pancreatic duct induces an increase of intrapancreatic pressure which initiates the lesions (Hyun and Lee 2014); such mechanism is not expected to operate during caerulein-induced inflammation. Inflammation is found in both models, with infiltration of neutrophils, known to produce high amounts of ROS responsible for acinar cell death. Relocalization of cathepsin B is a known feature of pancreatitis and is associated with intra-acinar activation of the trypsinogen and autodigestion of the gland. This colocalization has been well documented in caerulein-induced pancreatitis, but poorly in PDL with only few studies showing the relocalization of cathepsin B after PDL in rabbit and rat species (Ohshio et al. 1991, Hirano and Manabe 1993). However the PDL model is known to be associated with effects that may be species specific (Ohshio et al. 1991, Hyun and Lee 2014).

Both in the PDL and the caerulein model, ductal cells resist to destruction, unlike acinar cells, suggesting that the duct cells trigger the phenotypic changes observed in both models. This is consistent with our observation that injured ductal cells can induce ADM. It would be interesting to compare the initial events occurring following PDL or caerulein injections to better understand the molecular mechanisms that differ in both experimental settings.

All together, our results suggest that the ErbB pathway drives ADM and PanIN development in the caerulein model, and that other pathways, yet to be identified, are important for the development of these lesions during PDL.

C- Ductal deletion of Hnf6, a new model of duct dysmorphogenesis and pancreatitis

Embryonic deletion of Hnf6 in acinar and ductal cells using Pdx1-Cre mice leads to spontaneous pancreatitis (Zhang et al. 2009). This raises the question of whether deletion of Hnf6 in ductal or acinar cells induces pancreatitis. To answer this question, we deleted Hnf6 in acinar cells using *ElaCreER Hnf6^{ff}* mice. No pancreatitis developed in this context (data not shown), suggesting a role for the ductal deletion of Hnf6 as a pancreatitis inducer. Indeed, when Hnf6 was deleted in postnatal ductal cells using a *Sox9CreER*, lesions reminiscent of chronic pancreatitis with ADM developed within the pancreas; lipomatosis was also observed. This raises the question about the functional link between ductal defects and acinar injuries.

Various transgenic mouse models with defects in primary cilia displayed pancreatic injuries similar to those seen in the absence of Hnf6 in ducts (Cano et al. 2004, Zhang et al. 2005, Cano et al. 2006). As Hnf6 is involved in the regulation of cystogenic genes (Pierreux et al. 2006), we analyzed primary cilia in Hnf6-deficient dilated ducts. Using immunohistochemistry, we observed primary cilia with aberrant morphology. To our knowledge, our data are the first to link ductal ciliary defects to acinar lesions.

In kidney, defects in primary cilia induce cysts formation and several studies have tried to identify the underlying mechanisms. It was previously thought that perturbed flow sensing was the cause of abnormal proliferation and cyst formation, but subsequent studies have suggested that this might be not the primary pathogenic mechanism of cyst formation (Davenport et al. 2007, Piontek et al. 2007). In these studies, deletion of ciliary genes occurred in adult tissue and not during embryogenesis, leading to delayed development of cysts. This potentially means that the function of deleted proteins could differ in the adult and developing organ and the developmental state of the kidney could determine the response of the renal epithelium. Authors also suggest that primary cilia could be important for

maintenance of the planar cell polarity (PCP) and loss of this organelle could explain their phenotypes. In our model of postnatal Hnf6 deletion, dilated ducts never evolved to full-blown cysts. No proliferation was seen in dilated duct deficient in Hnf6 and no cysts were detected at 6 months of age, suggesting that ductal dilatation could potentially result from aberrant PCP. In the kidney, it has been shown that PCP is necessary to allow correct morphology and diameter of the nephrons. It was suggested that functional primary cilia support non-canonical signaling (PCP). If primary cilium is absent or non functional, fluid flow is not monitored and this induces a shift to canonical Wnt signaling (Simons et al. 2005, Berbari et al. 2009). We could then hypothesize that aberrant PCP signaling induces ductal dilatation by misoriented cell division as demonstrated in the kidney (Fischer et al. 2006, Happe et al. 2011). In a mouse model lacking Hnf1 β in the kidney, dilatation of collecting ducts occurs quickly after birth and is caused by loss of PCP (Fischer et al. 2006, Winyard and Jenkins 2011). Despite controversial studies about the role of cilia in the modulation of the Wnt pathway, it is likely that deregulation of Wnt signaling contributes to cyst formation.

As discussed below, in kidney tissue, defects in flow sensing does not seem to be the cause of cyst formation. We cannot exclude the possibility that in pancreatic ducts, ductal dilatation primarily results from perturbed flow sensing. In the vasculature, it is well known that shear stress regulates lumen diameter and this mechanism was also proposed to explain development of cysts in kidney cells (Nauli et al. 2003). More investigations are needed to identify the underlying mechanisms of ductal dilatation in pancreas.

Finally, why ductal dilatations are responsible for acinar injury remains an open question. Loss of primary cilia may induce ductal stress leading to the release of inflammatory molecules and further acinar injuries. Also, injured ductal cells may release exosomes. Exosomes are small extracellular vesicles released in the extracellular space and in the blood circulation. They contain proteins and nucleic acids (Kalluri 2016) and it has been shown that they can fuse to the plasma membrane or engage cell surface receptors, ultimately leading to release of their content (Colombo et al. 2014, Zhang et al. 2015). Their role in cancer has been

investigated and it has been demonstrated that the content of exosomes from cancer cells can modify the activity of recipient cells (Zhang et al. 2015). We could then hypothesized that exosomes released from ductal cells may transfer molecular signals to acinar cells, eventually leading to acinar injury.

Another hypothesis is based on the known capacity of primary cilia to secrete molecules (Kaimori et al. 2007, Avasthi and Marshall 2013). Indeed it has been shown that cilia from *Chlamydomonas* cells are able to secrete, in the extracellular environment, vesicles called ectosomes which contain biological effector molecules (Wood et al. 2013). Assuming that primary cilia from mammalian cells are also able to secrete ectosomes, it is conceivable that altered ciliary function can modify ectosome secretion and affect acinar homeostasis. It was also demonstrated that mammalian cells induce release of the ectodomain of the polyductin 1 protein by proteolytic cleavage similar to cleavage-dependent Notch ligand activity. The released ectodomain of the protein potentially maintains planar orientation of the target cells (Kaimori et al. 2007). Other proteins may be shed at the ciliary surface to ensure communication with other cells.

Finally, to identify the link between injured ductal cells and acinar lesions, we performed a microarray experiment on 3 week-old pancreata from controls and transgenic mice (data not shown). Unexpectedly this microarray did not reveal any increase in cytokines or inflammatory molecules in the pancreas of transgenic mice. Such molecules may have been missed in the experiment, since whole organ and not purified ductal cells were subjected to the microarray analysis, leading to dilution of the duct-emitted signal. Performing the microarray experiment on organoid cultures from ductal cells is expected to bypass this problem.

Our current results suggest that maintenance of ductal homeostasis is primordial for maintenance of acinar tissue but future investigations are needed to clarify the role of the primary cilia in this phenotype. In addition to the abovementioned functions as mechanosensors or PCP regulators, primary cilia are involved in the regulation of signaling pathways, such as Hedgehog (Berbari et al. 2009). Future experiments should address the functionality of signaling pathways,

as well as calcium transport to obtain clues about the link between primary cilia and acinar defects.

D- LKB1 in cilia and cyst formation

Inactivation of the serine/threonine kinase Lkb1 (Stk11) in postnatal ductal cells using Sox9CreER mice generated a phenotype similar to that obtained in the absence of Hnf6, namely lipomatosis, dilated ducts and development of ADM. In contrast to the phenotype obtained with Hnf6 deletion, increased proliferation of the ductal cells was observed in the absence of Lkb1. This could explain dilation of the ducts. In other organs, like in kidney, ciliary defects and presence of cysts and dilated ducts often coincide with increased cell proliferation, even if the role of proliferation as a driving force in dilation of ducts is still debated (Nauli et al. 2003, Fischer et al. 2006, Lantinga-van Leeuwen et al. 2007).

Another possible mechanism linking ductal dilatation with defects in primary cilia is the role of Lkb1 as a central regulator of cell polarity and energy metabolism (Granot et al. 2009, Sebbagh et al. 2009). In β -cells, Lkb1 deficiency leads to a 65% increase in cell volume and loss of cell polarity. In our model, we did not observe obvious apical polarity defects. However, since Lkb1 is also located in adherens junctions (Sebbagh et al. 2009), definitive conclusion about lack of apical polarity defects awaits analysis of the integrity of adherens junctions. In kidney, Lkb1 is located in primary cilia (Boehlke et al. 2010) and is involved in mechanosensory functions and in size regulation of kidney cells. In normal conditions, Lkb1 is localized in the axoneme and basal body, whereas its substrate, the AMP-dependent protein kinase (AMPK) is only found at the basal body. Upon cilium bending by fluid flow, Lkb1 is activated and transported from the axoneme to the basal body, where it phosphorylates AMPK. Thus the primary cilium is required for Lkb1 activity (Figure 1). In the absence of Lkb1, up-regulation of the mTORC1 pathway leads to an increase in the size of the cells and to cyst development. Lkb1 knock-down in Madin-Darby Canine Kidney (MDCK) cells did

not affect cilium formation (Boehlke et al. 2010), in contrast to our study. In another study, knock-down of Lkb1 in 3T3 cells resulted in diminished length of the cilia (Jacob et al. 2011), which is very similar to what we obtained in pancreatic ductal cells. The reasons of the discrepancy between cell and animal models are unclear, and it is likely that Lkb1 function varies from one cell type to the other. In some studies, the use of rapamycin, a specific inhibitor of mTORC1 pathway, led to regression of cysts in mouse models of polycystic kidney disease (Shillingford et al. 2006, Wu et al. 2009, Winyard and Jenkins 2011). In our Lkb1-deficient model, mTOR signaling deserves to be investigated to determine if it contributes to duct dilatation.

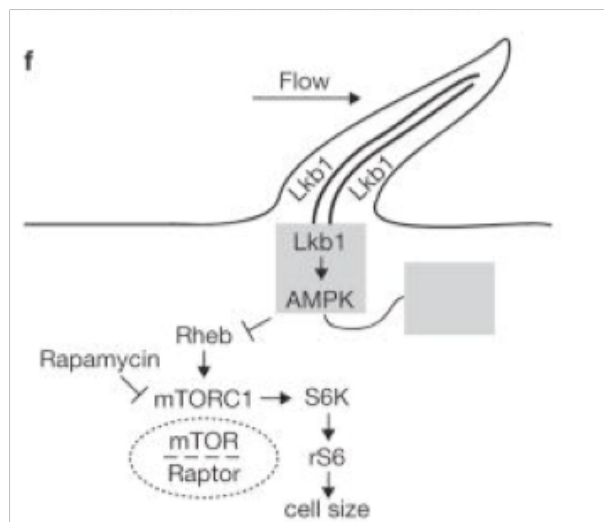


Figure 1. Schematic representation of mTOR signalling regulation in primary cilia (Boehlke et al. 2010).

Ductal dilatation or cyst formation are usually triggered by defective primary cilia. When Hnf6 or Lkb1 are deleted in pancreatic ducts, primary cilia have swollen

tips, leading us to hypothesize a defective retrograde transport, as seen in other studies (Hurd et al. 2010, Ocbina et al. 2011, Qin et al. 2011). Two IFT complexes mediate intraflagellar transport, IFT-A (retrograde transport) and IFT-B (linked to anterograde transport). Deletion of the IFT-B complex abrogates cilia formation whereas disruption of the retrograde transport (IFT-A) leads to swelling of the ciliary tips (Goetz and Anderson 2010). It was previously demonstrated that loss of some IFT retrograde proteins could induce swelling and abnormal Shh signaling. For example, abnormal structure of primary cilia with large tip swellings were detected following knockdown of Retinitis Pigmentosa 2 (RP2) protein, which together with polycystin 2 belongs to a calcium sensitive complex. This swelling is likely caused by aberrant trafficking of cilia proteins (Hurd et al. 2010). Deletion of IFT122, which belongs to the IFT-A complex, also results in perturbed retrograde transport with accumulation of IFT proteins in swollen cilia tips. Moreover, IFT122 plays a crucial role in regulating the transport of Gli2 and Gli3 as mutation of this protein leads to accumulation of Gli2 and 3 at the ciliary tip, demonstrating a role for IFT122 in the regulation of Shh pathway (Qin et al. 2011). Finally, mutations of the heavy chain of the cytoplasmic dynein-2 motor (DYNC2H1) also disrupts the Shh signaling pathway with accumulation of Smo, Ptch1 and Gli2 at the cilium tip (Ocbina et al. 2011).

We are currently investigating the expression of retrograde proteins in Lkb1 and Hnf6-deficient models to confirm the possibility that it causes swelling and ciliary dysfunction. In addition since defects in retrograde transport is linked to perturbed Shh activity, and since this pathway is often deregulated in cancer (Goetz and Anderson 2010), testing the functionality of the Shh pathway is a priority for future work.

E- Primary cilia and cancer

No functional link between cancer and Lkb1 has yet been identified, but human patients with Peutz-Jeghers syndrome (PJS) have an increased risk to develop PDAC. To clarify the role of Lkb1 in pancreatic cancer, we have recently combined deletion of Lkb1 and activation of oncogenic Kras in pancreatic ducts. In this model, the mice developed large cysts and Intraductal Papillary Mucinous Neoplasms (IPMN) (L. Collet, personal communication). These data open perspectives for the study of primary cilia in ductal lesions of PJS patients.

Our results raise the possibility that perturbed duct homeostasis may trigger tumorigenesis from acinar cells that harbor a Kras mutation. Indeed, we have observed that ductal deletion of Hnf6 and Lkb1 can induce acinar expression of a member of the ErbB pathway which is required for PanIN development. In support of this hypothesis, acinar-derived PanIN lesions are frequently associated with duct-derived IPMN (Maire et al. 2013). Combining Cre-mediated Lkb1 or Hnf6 deletion in the ductal cells and Kras^{G12V} induction mediated by Flp recombination in the acinar cells, would allow to test this hypothesis.

To further support a functional link between primary cilium and cancer, we looked for ciliary genes in genomic studies dedicated to identify genes with copy-number variation in patients with familial history of PDAC (Lucito et al. 2007, Al-Sukhni et al. 2012). We found three genes associated with primary cilia. Intraflagellar transport protein 80 homolog (IFT80) is localized in IFT-B (linked to anterograde transport), and is necessary for maintenance and function of primary cilia (Yang and Wang 2012). ExoC4 is a protein of the exocyst complex involved in basolateral protein sorting and membrane trafficking (Kang and Folsch 2009). DOCK1 was identified by both studies and is associated with the ELMO-DOCK1 (DOCK180) complex. This complex serves as a bipartite guanine nucleotide exchange factor for Rac1, a GTPase whose absence has been associated with abnormal primary cilia (Epting et al. 2015).

Several studies addressing a wide range of cancers have shown loss of primary cilia, including in pancreatic cancer resulting from somatic mutations or genome dysregulation (Hassounah et al. 2012, Shpak et al. 2014). Changes in the expression level of ciliary genes have also been found, but it remains to be determined if they are a consequence or a cause of the tumorigenic process. Further investigations are needed to clarify the role of this organelle in pancreatic tumorigenesis

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IX. APPENDIX

Extraction of High-quality RNA from pancreatic tissues for gene expression studies

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Extracting good quality RNA from pancreas is challenging given the presence of high amounts of RNAses in this tissue. In this technical paper we describe an improved method to extract high quality pancreatic RNA. This method proved useful for our laboratory and for collaborators, and will be implemented in future projects. I contributed to the study concept and design, acquired and analysed data and also participated to the writing of the manuscript.

Abstract

Extracting RNA from pancreatic tissue is notoriously challenging because of the organ's high RNase content. Standard methods using TriPure or TRIzol classically yield RNA of sufficient quality for routine gene expression analysis but not for microarray or deep sequencing analysis. Here we developed a simple method to extract high-quality RNA from mouse pancreas. Our method uses an RNase inhibitor and combines different protocols using guanidium thiocyanate-phenol extraction. It enables reproducible isolation of RNA with an RNA integrity number around 9.