Pancreatic acinar cell plasticity

Senescence, epithelial-mesenchymal transition and p53

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Abstract

Pancreatic acinar cells display plasticity to acquire distinct differentiation programs, being involved in diseases as chronic pancreatitis and pancreatic ductal adenocarcinoma. This work shows that acinar cells cultured in suspension undergo dedifferentiation, acquiring a pancreatic embryonic progenitor phenotype. Dedifferentiated cells turn on a senescent program, associated with activation of p53 and Ras pathways. A similar progenitor-like phenotype with activation of senescence is present in experimental chronic pancreatitis. Acinar cultures lacking p53 overcome growth arrest and lose the pancreatic phenotype, undergoing an epithelial-mesenchymal transition, while maintaining the expression of pre-pancreatic endoderm and stem cell markers. In experimental acute pancreatitis, absence of p53 results in increased acinar cell proliferation and delayed regeneration. Our findings support a role for acinar cell dedifferentiation in the initiation of pancreatic diseases. A p53-dependent control of cell growth and epithelial differentiation constitutes a tumor suppressive mechanism that may limit PDAC development.

Resumen

Las células pancreáticas acinares poseen plasticidad que les permite adquirir distintos programas de diferenciación, estando implicadas en enfermedades como la pancreatitis crónica y el adenocarcinoma ductal pancreático. En este trabajo hemos demostrado que las células acinares cultivadas en suspensión se desdiferencian, adquiriendo un fenotipo de progenitores pancreáticos embrionarios. En estas células se induce un programa de senescencia asociado con la activación de las vías de p53 y Ras. Un fenotipo similar se evidencia en modelos de pancreatitis crónica experimental. Cultivos acinares en los que se ha inactivado p53 sobrepasan el bloqueo de crecimiento y pierden el fenotipo pancreático, presentando una transición epitelio-mesenquimal y manteniendo la expresión de marcadores de endodermo pre-pancreático y de células madre. Durante la inducción de una pancreatitis aguda experimental, la ausencia de p53 resulta en un incremento de la proliferación acinar y en un retraso en la regeneración. Nuestros resultados demuestran que la desdiferenciación de las células acinares participa en el desarrollo de enfermedades pancreáticas. El control del crecimiento celular y de la diferenciación pancreática epitelial dependiente de p53 constituye un mecanismo de supresión tumoral que puede limitar el desarrollo del PDAC.

Preface

Pancreatic acinar cells have been previously shown to possess the ability to reprogram into other differentiated cell types. Furthermore it has been demonstrated that acinar cells can be in the origin of chronic pancreatitis and pancreatic ductal adenocarcinoma (PDAC). PDAC is an important health problem in developed countries, being the fifth cause of cancer related deaths in Europe and having a very bad prognosis with a 5-year survival rate of <5%. Since chronic pancreatitis is an established risk factor for PDAC, it is important to better understand the mechanisms involved in pancreatic acinar cell differentiation, which may be implicated in the development of pancreatitis and consequently in the development and progression of PDAC.

Our work has brought new insights into the plasticity potential of pancreatic acinar cells, showing that these cells have undergo dedifferentiation to a pancreatic progenitor-like phenotype upon suspension culture or after the induction of chronic pancreatitis. We have also shown that activation of senescence constitutes an important tumor suppressive mechanism that blocks the growth of acinar cells undergoing dedifferentiation both *in vitro* and *in vivo*. Moreover, we find that the growth arrest is dependent of p53 and we have demonstrated a new function of this tumor suppressor in inhibiting EMT and the acquisition of stemness traits in normal pancreatic epithelial cells. Considering that p53 is often mutated in PDAC, our results provide clues on how the inactivation of p53 may contribute to tumoral development and dissemination and how strategies aimed at the reactivation of this pathway may provide future therapies to fight PDAC.

Index

Abstract i

Resumen ii

Preface iii

Index iv

INTRODUCTION 1

I. Pancreas Biology 3

- I.1. Pancreas anatomy and physiology 3
- I.2. Pancreatic development in the mouse 6
- I.3. Pancreatic acinar cells 16

II. Pancreatitis 23

- II.1. Acute pancreatitis 23
- II.2. Chronic pancreatitis 25
- II.3. The etiology of pancreatitis 27
- II.4. Pancreatic stellate cells: the role in pancreatitis 31
- II.5. Experimental models of pancreatitis 33

III. Pancreatic Ductal Adenocarcinoma 37

- III.1. PDAC pathology 37
- III.2. PDAC epidemiology 39
- III.3. Molecular genetics and PDAC progression model 43
- III.4. Genetic approaches to model PDAC in mice 52
- III.5. Pancreatic cancer stem cells 59
- III.6. Uncommon pancreatic tumors 60

IV. The tumor suppressor p53 65

- IV.1. Introduction 65
- IV.2. The p53 gene family 66
- IV.3. Regulation of the p53 pathway 70
- IV.4. The tumor suppressor functions of p53 74
- IV.5. p53 mutations and cancer 85

IV.6. p53 in development and cell differentiation 89

V. Epithelial-mesenchymal transition 93

V.1. The regulation of the EMT process 93

V.2. EMT in development and injury 99

V.3. EMT in cancer 100

HYPOTHESIS & AIMS 105

RESULTS 109

I. Research Publication 1 111

Adult pancreatic acinar cells dedifferentiate to an embryonic progenitor phenotype with concomitant activation of a senescence programme that is present in chronic pancreatitis

I.1. Supplementary Material 113

II. Research Publication 2 137

p53-dependent regulation of growth, epithelial-mesenchymal transition and stemness in normal pancreatic epithelial cells

II.1. Supplementary Material 139

II.2. Appendix 143

Caerulein-induced acute pancreatitis in $p53^{-/-}$ mice

DISCUSSION 153

I. Adult pancreatic acinar cells dedifferentiate into pancreatic progenitor-like cells 155

II. Dedifferentiated acinar cell cultures turn on a senescence program, with activation of the p53 and Ras pathways 158

III. Acinar cell dedifferentiation and senescence: implications for chronic pancreatitis and PDAC 161

IV. Cell cycle arrest in dedifferentiated acini is dependent of p53 165

V. Lack of p53 induces an EMT in pancreatic epithelial cells 167

VI. Pancreatic acinar cell cultures lacking p53 have increased stemness properties 170

VII. p53-null mice have a delay in acinar cell regeneration and increased acinar cell proliferation upon induction of injury 172
VIII. p53: a barrier to pancreatic acinar cell plasticity and to PDAC initiation 175

CONCLUSIONS 179

BIBLIOGRAPHY 183

ANNEX 211

Preliminary Results 213

INTRODUCTION

I. Pancreas Biology

I.1. Pancreas anatomy and physiology

The pancreas is a glandular organ composed of two distinct compartments, exocrine and endocrine, whose function is to regulate essential physiologic functions as food digestion and glucose homeostasis.

Ancient anatomists regarded the pancreas as an unusual organ, with no cartilage or bone and hence, the name derives from the Greek roots 'pan' meaning 'all' and 'creas' meaning 'flesh' [1].

In humans, the pancreas forms a well defined organ of 70-150 grams measuring 15-25 cm in length and the terms head, neck, body and tail are used to designate regions of the organ from proximal to distal. In rodents the shape of the pancreas is less well defined. The organ is localized in the upper region of the abdomen, connected to the duodenum by the ampulla of Vater, where the main pancreatic duct joins with the common bile duct [2].

I.1.1. The exocrine pancreas

The exocrine pancreas accounts for more than 90% of the whole organ and is composed of two main types of cells, acinar and ductal.

Acinar cells have a secretory function which is to produce hydrolytic digestive enzymes at a large scale. Acinar cells are organized into functional units called acini, have a pyramidal shape with a basal nucleus, regular arrays of rough endoplasmic reticulum, a prominent Golgi complex and numerous zymogen granules, containing the enzymes and located in the apical pole. There are at least 22 different digestive enzymes, many of which are secreted as inactive precursors becoming activated after entering the duodenum. Secretion of the pancreatic juice is regulated by hormones

such as secretin, cholecystokinin and gastrin, and also by neural stimuli [2-4]. The biology of acinar cells will be discussed in more detail in a subsequent chapter.

The ductal cells form a branched network, which delivers the pancreatic juice into the gastrointestinal tract. These cells are also responsible for the production of bicarbonate that neutralizes stomach acidity and mucins. Ductal cells of intercalated ducts form a simple squamous epithelium and are surrounded by little connective tissue. As ducts become larger the epithelium becomes either cuboidal or columnar and in the largest branches of the network, goblet cells are intermingled with ductal cells, forming about 2% of its structure. The intercalated ducts, which drain the acini, converge to form interlobular ducts that connect the different lobes of the pancreas, merging into the main pancreatic duct. The main pancreatic duct is formed by a columnar epithelium, surrounded by abundant connective tissue [2, 5].

At the junction between acinar cells and the adjacent terminal ductal epithelium are centroacinar cells with a low cuboidal shape and rich in mitochondria [2]. This cell type is very poorly characterized and it is uncertain whether centroacinar and terminal duct cells represent two different cell types or are functionally equivalent [6]. Centroacinar cells have been shown to display active Notch signaling [7]. Rovira et al were able to isolate a population of centroacinar and terminal ductal cells from adult mouse pancreas, characterized by high levels of Aldh1 enzymatic activity. The Aldh1+ population has increased progenitor properties, being able to form self-renewing “pancreatospheres” in suspension and displaying spontaneous endocrine and exocrine differentiation capacity [6].

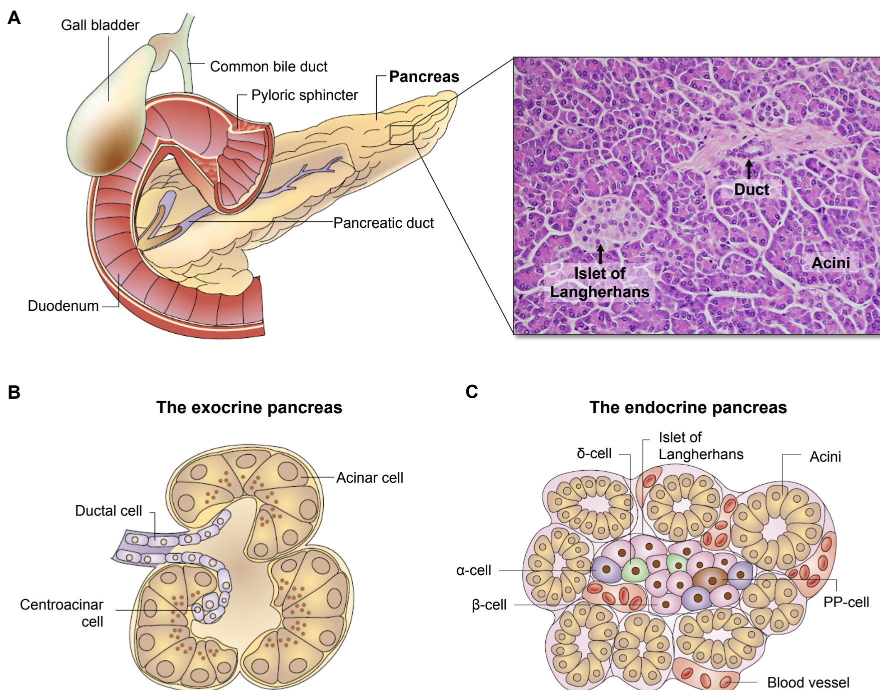


Figure I1. Anatomy of the pancreas. A. Gross anatomy of the pancreas (left). Hematoxylin and eosin staining of the pancreas showing the exocrine (Duct and Acini) and the endocrine compartments (Islet of Langherhans). **B.** Schematic representation of the exocrine tissue**. C.** Schematic representation of the endocrine tissue showing an Islet of Langherhans embedded in exocrine tissue. *Adapted from Bardeesy and DePinho, 2002* [8].

I.1.2. The endocrine pancreas

The endocrine pancreas is responsible for the regulation of metabolism and glucose homeostasis through a finely tuned control of hormone release. This compartment is formed by polyclonal structures called islets of Langerhans, which are compact cell clusters embedded in the exocrine tissue [2, 9]. There are five types of endocrine cells, each producing a different type of hormone. The β-cells secrete insulin and an insulin antagonist called

amylin, constituting the majority of cells in the islets. The α-cells secrete glucagon, the δ-cells secrete somatostatin, the PP-cells secrete pancreatic polypeptide, and the ε-cells produce ghrelin [10]. In rodents there is quite a sharp segregation within the islets such that the β-cells lie in the centre and the other types at the periphery, while in humans this segregation, although present, is less clear-cut [2, 9].

I.2. Pancreatic development in the mouse

I.2.1. Morphogenesis

Cells fated to form the pancreas arise from the primitive gut tube, derived from definitive endoderm. The first morphological evidence for a pancreatic domain appears as a thickening on the dorsal side of the foregut epithelium at embryonic day 9.5 (E9.5) in mice and on the 26th day of gestation in humans. Simultaneously, two ventral buds originate laterally within the epithelium, of which one regresses prior to gut rotation. After gut rotation, the dorsal and ventral buds fuse, around E12.5 in the mouse. At the same time, the pancreatic epithelium branches into the surrounding mesenchyme producing a highly proliferative population of multipotent progenitor cells with the ability of forming all pancreatic lineages. From E12.5 to E14 multipotent cells present at the tips of the branching epithelium directly produce pre-acinar cells that remain in the tips and bipotent duct/islet cells that populate the branches. Specification of all differentiated cell types that form the adult organ occurs by E15.5 and between this moment and birth, the differentiated cells undergo additional growth and maturation [4, 9, 11]. Human embryonic development of the pancreas is thought to go through similar steps.

I.2.2. Signaling during early steps of pancreas formation

The pancreas derives from the endodermal epithelium, being specified under the influence of inductive signals from the surrounding mesenchyme.

The ventral pancreas and the liver arise from common bi-potential progenitors from the lateral endoderm domain [12]. Initially, suppression of Wnt and fibroblast growth factor 4 (FGF4) signaling in the foregut induces liver and pancreas fates while active mesodermal Wnt signaling in the posterior gut suppresses these fates [13, 14]. Retinoic acid establishes an anterior-posterior positioning of the liver and pancreas domains within the gut [15]. FGF signals from the cardiac mesenchyme and bone morphogenetic protein (BMP) from the septum transversum mesenchyme coordinately induce the liver program and suppress the pancreas program. During foregut closure, lateral ventral endoderm cells move away from these signals and initiate ventral pancreatic development [16].

Patterning of the dorsal foregut is mediated by signals from the notochord and dorsal aorta. TGF-β/activin and FGF10 coming from the mesenchyme suppress sonic hedgehog (Shh) signaling within the endoderm, allowing the pancreatic program [17, 18]. Notch signaling is also activated by mesenchymal FGF10 and is responsible for maintaining progenitor cells in an undifferentiated state [19, 20].

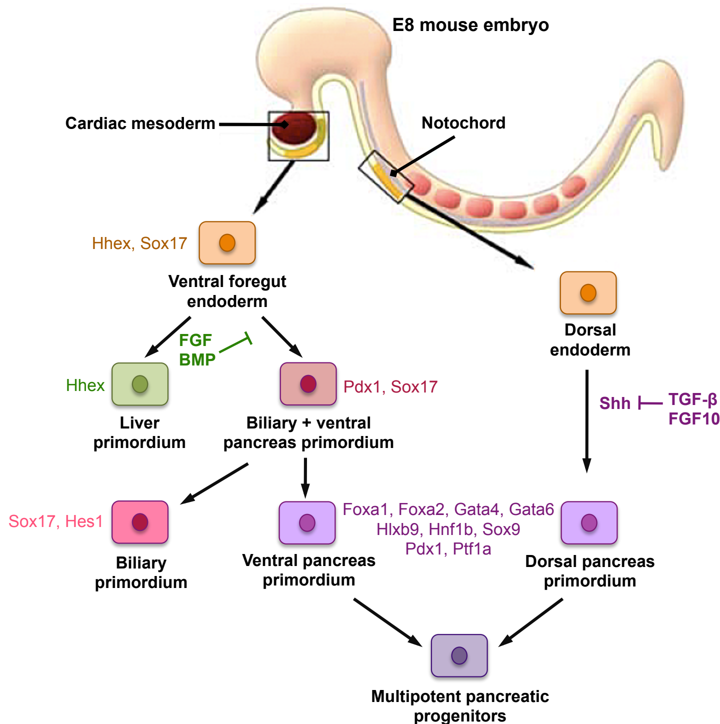


Figure I2. Early foregut endoderm patterning. Sagital view of an E8 mouse embryo, showing the dorsal and ventral pre-pancreatic endoderm. Signaling from the adjacent mesodermal derivatives, such as the cardiac mesoderm and the notochord, establishes the pancreatic domain. Distinct sets of signaling molecules and transcription factors control the specification of the pancreas, liver and billiary primordia in the ventral endoderm and of the pancreas primordium in the dorsal endoderm. *Adapted from Pan and Wright, 2011* [21].

I.1.2. Pancreatic multipotent progenitor cells

The newly specified pancreatic endoderm is initially marked by the expression of two transcription factors which are crucial for pancreatic development: the pancreas and duodenal homeobox gene-1 (Pdx1) and the pancreatic transcription factor-1 (Ptf1a/p48).

Pdx1 is first expressed around E8.5 during mouse development. Mice in which this gene has been inactivated, completely arrest pancreas

organogenesis [22, 23]; lineage tracing analysis has revealed that Pdx1 positive cells contribute to all pancreatic fates [24]. In the adult, Pdx1 is most highly expressed in the endocrine β- and δ-cells, having a low expression in exocrine cells. Pdx1 is required for appropriate β-cell function and its inactivation in β-cells leads to diabetes [25, 26]. It has also been shown to be essential in humans, where haploinsufficiency leads to maturity onset of the young (MODY4) [27].

Ptf1a / p48 expression is activated shortly after Pdx1, appearing at E9.5 in mice development. Ptf1a is required for the evagination of the ventral bud and the growth of the dorsal bud. Mice null for Ptf1a are devoid of all exocrine and most endocrine tissue, exhibiting postnatal lethality due to central nervous system problems [28, 29]. In the adult, Ptf1a expression is restricted to the exocrine pancreas being one of the main transcription factors regulating acinar cell differentiation (discussed below).

Additional transcription factors also have a role in regulating the patterning of the gut endoderm, being required for the establishment of the pancreatic fate:

Hlxb9 activates the expression of Pdx1 and is essential for dorsal bud formation and later for β-cell development [30, 31].

Sox17 is expressed, together with Pdx1, in the ventral endoderm that gives rise to both biliary and pancreatic primordia. Persistent Sox17 expression leads to billiary tract specification whereas its downregulation is required for pancreas formation [32].

Hhex is expressed in the ventral-lateral foregut, which gives rise to pancreas and liver. Hhex is required for the correct location of pancreatic progenitors in the leading edge of the ventral embryonic endoderm; in Hhex-null mice there is no ventral induction of Pdx1 or Ptf1a [16].

Hnf6 / Onecut1 is a key determinant of pancreas specification, regulating the timing of Pdx1 expression [33]. Hnf6 is also required for endocrine progenitors formation [34, 35] and for normal duct cell development [36].

Foxa1 / HNF3a and **Foxa2 / Hnf3b** are required for pancreas formation by specifying the pre-pancreatic endoderm through induction of Hnf6 and Pdx1 [37, 38]. Later on, Foxa2 is necessary for the maturation of endocrine cells[39, 40].

Gata4 and **Gata6** are expressed in most cells of early pancreatic epithelium. To circumvent the early lethality of the constitutive null animals for either of these proteins, Gata4 or Gata6 were fused to an engrailed repressor domain and targeted specifically to the pancreas. Expression of Gata6-engrailed under a Pdx1 promoter led to severe pancreas disruption or agenesis while Gata4-engrailed mice were normal [41]. A second approach was to use tetraploid embryonic stem cell aggregation to generate Gata4 and Gata6 null mice. In both cases, disruption or absence of the ventral pancreas was seen, revealing that these genes are required for pancreas formation [42] .

Several other transcription factors are expressed in multipotent pancreatic progenitor cells, being essential for appropriate pancreas differentiation:

Hnf1b / vHNF1 / TCF2 is essential for the formation of both pancreatic buds, as it activates Pdx1 ventrally and Hnf6 and Ptf1a in both buds [43]. Lineage tracing experiments have shown that it is expressed in a subpopulation of pancreatic multipotent cells that can give rise to all pancreatic lineages [44].

Sox9 is expressed during pancreatic development and it has been described to label multipotent pancreatic progenitor cells [45-47]. It was shown to be essential for the persistence of early pancreatic progenitors, by stimulating

their proliferation and survival [48]. Together with Hnf1b and Hnf6, Sox9 reinforces the transcriptional network controlling the formation of Ngn3 positive endocrine progenitor cells [49].

Myc is expressed in multipotent pancreatic progenitor cells [50] and its inactivation during pancreas development leads to a great reduction in the number of acinar cells and to poorly branched ducts but it does not affect the endocrine compartment [51, 52].

Hes1 is a downstream effector of the Notch pathway and is expressed in multipotent progenitor cells, being necessary for their maintenance in an undifferentiated state and for their expansion [53-55]. Later in development Hes1 controls the boundary between the acinar precursors located in the tips and the bipotent ductal/endocrine trunk cells (discussed bellow).

Rbpj has a dual role in early pancreatic development and Rbpj deficient pancreata present multiple defects. As a transcriptional mediator of Notch signaling it is necessary for the expansion of the pool of undifferentiated progenitor cells, suppressing premature endocrine differentiation [56, 57]. As a critical subunit of the PTF1 complex, together with Ptf1a, it is needed to maintain the commitment of the nascent epithelium to pancreatic development, including the extensive early growth [58, 59].

The establishment of a pancreatic progenitor cell signature has been a matter of great interest and work by Zhou et al. has been critical to identify the multipotent progenitor cell population. By using *in vivo* genetic lineage tracing they have revealed that cells present at the tips of the expanding epithelium by E12.5 are multipotent pancreatic progenitor cells (MPPCs) capable of contributing to all the differentiated lineages of the pancreas (acinar, ductal and endocrine) and characterized by co-expressing Pdx1, Ptf1a, c-myc and Carboxypeptidase A [50].

Recent work by Solar et al., has shown that Hnf1b also marks pancreatic multipotent cells up to E13.5 and revealed that during a short period both Cpa-, Hnf1b+ trunk cells and Cpa+ tip cells are MPPCs [44].

I.2.3. Specification of the pancreatic lineages

By mid pancreatic development (around E14 in the mouse) the multipotent progenitor population becomes depleted and cell differentiation begins through a phase of intense acinar, ductal and endocrine cell formation. This period has been termed secondary transition and is characterized by the appearance and dramatic increase of digestive enzymes and endocrine hormones such as insulin and glucagon. The term has been created to distinguish this phase from the early primary transition where the first endocrine cells, predominantly glucagon positive, are formed [60].

At this stage, pancreatic tissue has a complex epithelial architecture with two main developmental compartments reminiscent of a tree, with tips - containing the fast replicating pre-acinar cells - and trunks - containing bipotent ductal/ endocrine cells [3].

Ptf1a is essential for acinar cell differentiation and its super-induction at the MPPCs located at the tips directs them towards an acinar fate [53, 61]. In acinar progenitor cells, Ptf1a promotes the induction of **Nr5a2**, which together, specify the acinar fate; the PTF1 complex activates the expression of the acinar-specific transcription factors **Rbpjl** and **Mist1** [3]. The acinar specific program will be discussed in further detail in the next section.

At the same time, **Nkx6.1** and **Nkx6.2** are expressed in the trunk where they antagonize the influence of Ptf1a, thus inhibiting acinar fate and directing these cells into a bipotent endocrine-ductal fate [62]. **Prox1** is expressed in a

similar fashion as Nkx6.1 and has also been shown to suppress the acinar fate in the trunk cells [63].

During the secondary transition, **Hes1** expression also remains restricted to the trunk cells where it seems to mediate Notch signaling suppression of the acinar fate, through inhibition of Ptf1a [53, 64].

Cells located in the trunk epithelium may give rise either to ductal or endocrine cells, as recently shown by lineage tracing experiments [44, 65]. Scattered within the tubule epithelium are precursor cells that initiate the expression of Neurogenin 3 (**Ngn3**), the key transcription factor that commits these cells to the endocrine fate [4]. To date, no analogous transcriptional regulatory factor that commits precursor cells to the ductal lineage has been identified and ductal development may be the default option for pancreatic cells that do not activate either Ptf1a or Ngn3 [3].

Despite the fact that a master regulator specifying ductal fate has not been identified, Hnf proteins have been shown to play an important role in ductal development.

Hnf6 / Onecut1 is present throughout the early pancreatic bud, subsequently maintained in mature acinar and ductal cells, and decreased in endocrine cells, and it is particularly important for proper duct cell development [36]. In the absence of Hnf6, acinar tissue forms nearly normally but the endocrine cells born during the secondary transition do not appear and the ducts are dilated and cystic. Hnf6 inactivation during pancreatic development leads to an increased number of ducts, ductal metaplasia and cysts, histological features reminiscent of pancreatitis [35, 66].

Hnf1b / vHNF1 / TCF2 is expressed in the early embryonic pancreatic epithelium and becomes restricted to centroacinar cells and ductal cells in the adult. Early Hnf1b+ precursor cells give rise to all the differentiated

cell-types in the mature pancreas but the differentiation potential of Hnf1b-expressing cells becomes more restricted at later stages of development [44]. Hnf1b controls the battery of genes required for the formation of primary cilia in the kidney [67] and its expression is absent in the defective ducts of Hnf6-deficient embryonic pancreas, suggesting that the absence of cilia in these mice is due to the loss of Hnf1b [66].

Within the endocrine cell compartment, **Ngn3** resides at the top of the transcription factor hierarchy and all pancreatic endocrine cells go through an $Ngn3^{+}$ stage [4]. Ngn3 is essential for endocrine cell formation and animals lacking this gene are devoid of islets and die soon after birth due to hyperglycemia [24, 68]. Downstream of Ngn3 there are several transcription factors that regulate the formation of the various cell types within the islet. **NeuroD**, **Isl1**, **Pax**, **Nkx**, **Arx** and **Irx** genes all contribute to maintaining the appropriate balance of the different types of endocrine cells [69]. For instance, inactivation of NeuroD, Isl1 or Pax6 essentially eliminates endocrine formation and loss of Nkx2.2 leads to a complete absence of β-cells, with a reduction in α and PP cells [70]. Similarly, Nkx6.1 depletion results in a significant loss of β-cell mass [71].

The **Maf** family of basic-leucine-zipper transcription factors has also an important role in β-cell formation and function [72]. MafB is expressed in α- and β-cells early during embryogenesis. As development proceeds, $MafB^{+}/Nkx6.1^{+}$ cells take on a β-cell identity and $MafB^{+}/Nkx6.1^{-}$ acquire an α-cell fate. In developing β-cells, a switch from MafB to MafA directly regulates insulin transcription, thus playing a central role in β-cell maturation [73]. As the Maf proteins, several endocrine transcription factors remain expressed in adult islets, playing critical roles in both formation and maintenance of function of the endocrine compartment during adulthood [4].

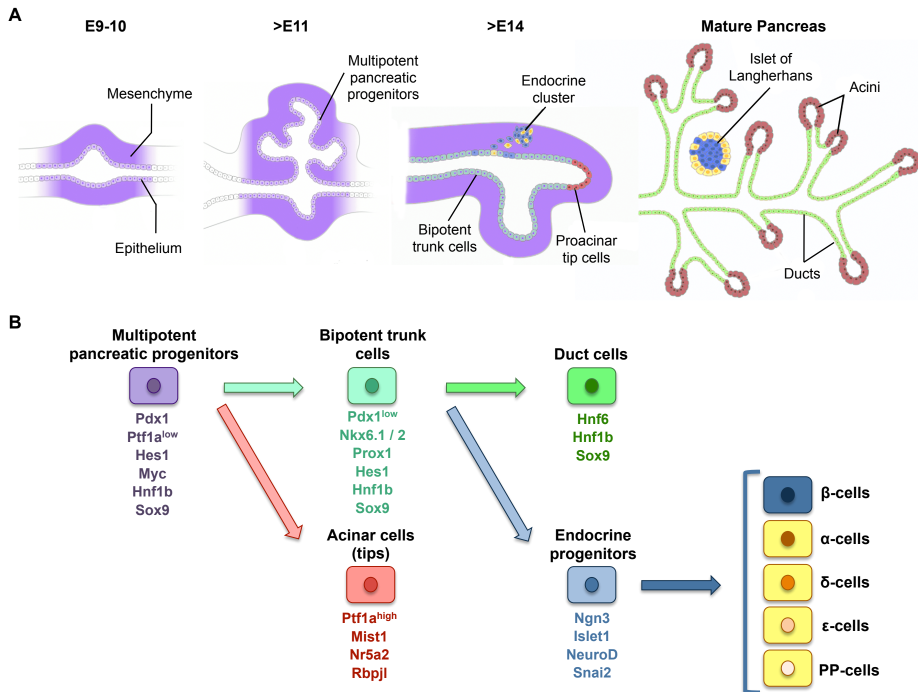


Figure I3. Pancreas development. A. Pancreas organogenesis. Pancreas development starts at approximately E9, when the pre-pancreatic epithelium thickens and evaginates forming a protuberance that bulges into the surrounding mesenchyme (**E9-10**). From E9, the pancreatic epithelium undergoes extensive proliferation and branching morphogenesis (**>E11**) and on E12.5 occurs the fusion of the ventral and dorsal buds. Around E14, the pancreatic multipotent progenitor cells become depleted and cellular differentiation begins. Cells located in the tips become committed to become acinar, while cells located in the trunks are bipotent, giving rise to both endocrine and ductal cells. Endocine progenitor cells scattered throughout the epithelium delaminate into the surrounding mesenchyme, assembling into endocrine clusters (**>E14**). At birth, the exocrine pancreas is formed by the acini (organized units of differentiated acinar cells) and by the ductal network. The endocrine pancreas is organized into islets of Langherhans, which comprise the β-cells, forming the core of the islet, and the α, δ, PP and ε-cells, located in the periphery of the islet (**Mature pancreas**). **B.** Important transcription factors that are expressed at each stage of pancreas development and which determine the specification of the distinct pancreatic cell lineages. *Adapted from Cano et al, 2007* [11].

I.3. Pancreatic acinar cells

The pancreatic acinar cell is a highly specialized structure developed for synthesis, storage, and secretion of digestive enzymes. Consequently, acinar cells express high levels of transcription factors that regulate processes which are specific of secretory cells [3].

I.3.1. The PTF1 complex

Ptf1a is a bHLH transcription factor that requires the formation of a trimeric complex (PTF1) for its activity. This complex contains a ubiquitous bHLH co-factor to bind DNA, such as E12, and a third DNA-binding subunit that binds specific TC-rich sequences [58]. In early pancreatic development, the third subunit is Rbpj / Rbpjk /CSL /Cbf1 and the trimeric complex is designated embryonic-type PTF1 (ePTF1). Although Rbpj is a known transcriptional mediator of the Notch pathway, its function in PTF1 is independent from its role in Notch signaling. The ePTF1 complex recognizes an extended DNA sequence containing an E-box, to which Ptf1a and the second cofactor bind, and a TC-box, to which Rbpj binds [3]. Masui et al have shown that the formation of the PTF1-J complex is necessary for the early developmental functions of Ptf1a, as animals with a mutation in Ptf1a that impedes the binding to Rbpj have a phenotype similar to Ptf1a null mice [59].

The onset of acinar differentiation is marked by a change in the subunit composition of the PTF1 complex. In adult acinar cells the predominant form of PTF1 contains Rbpjl, rather than Rbpj [58], indicating that the complex containing Rbpjl has specific functions in the mature acini. Rbpjl is a closely related Rbpj paralogue that arose by gene duplication [74] and the adult-type PTF1 (aPTF1) complex recognizes the same DNA sequence as the Rbpj-containing complex [58].

Rbpjl first appears during pancreatic development in early acinar cells, shortly after the superinduction of Ptf1a expression in tip progenitor cells during the secondary transition. Activation of Rbpjl transcription requires the binding of the ePTF1 complex to the Rbpjl promoter. It has been shown by ChIP analysis performed using embryonic pancreas that as Rbpjl accumulates during acinar cell maturation, it gradually replaces Rbpj in the complex. Once activated by the ePTF1 complex, auto-activation via the aPTF1 complex maintains Rbpjl transcription [75].

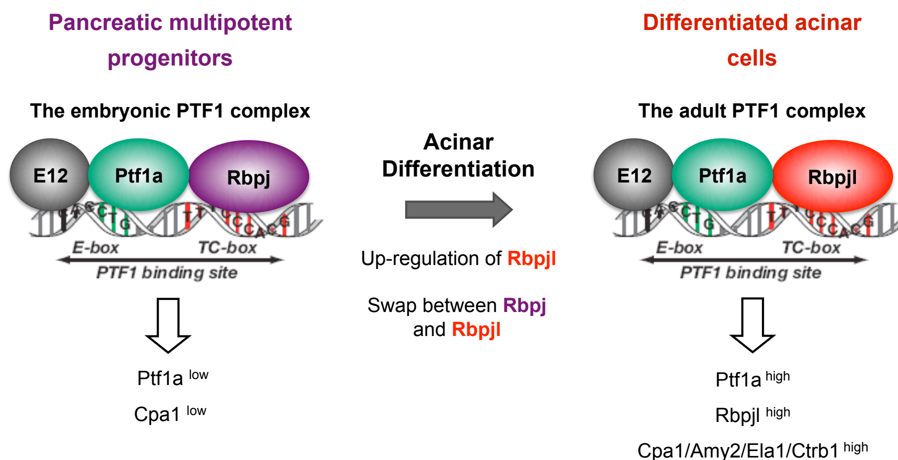


Figure I4. The PTF1 complex. In multipotent pancreatic progenitor cells, the embryonic form of the PTF1 complex (ePFT1) is constituted by Ptf1a, an ubiquitous bHLH co-factor (i.e. E12) and Rbpj. During development, when acinar cell specification occurs, the ePTF1 complex binds to Rbpjl promoter. During acinar cells maturation, Rbpjl starts to accumulate, replacing Rbpj in the adult form of the PTF1 complex (aPTF1). aPTF1 promotes complete acinar differentiation, enhancing the expression of acinar specific genes, such as the digestive enzymes Caboxypeptidase A1 (Cpa1), Amylase 2 (Amy2), Elastase 1 (Ela1) and Chymotripsinogen B1 (Ctrb1). *Adapted from Masui et al, 2007* [59].

Acinar genes requiring the Rbpjl form of PTF1 for optimal expression were identified in Rbpjl-deficient mice. The pancreas of Rbpjl null mice is one-third smaller than normal due to smaller, incompletely differentiated, acinar cells. Deep sequencing analysis of the mRNA from Rbpjl-null E17.5 embryos identified only 53 genes dependent on the Rbpjl-form of the PTF1 complex while, for the vast majority of PTF1 gene targets, it appears that Rbpj compensates for the absence of Rbpjl [76]. The aPTF1 dependent gene set encodes 27 of the 28 major acinar secretory proteins, genes involved in mitochondrial carbon, nitrogen and energy metabolism, creatine biosynthesis, and components of the intracellular protein transport apparatus. Thus, the role of the aPTF1 seems to be very selective in promoting complete acinar cell differentiation by enhancing secretory cell functions [76].

I.3.2. The acinar specific program

The transcription factor Mist1/Bhlhb15 constitutes another key regulator in the terminal differentiation of acinar cells. Mist1 is highly expressed in developing and mature pancreatic acinar cells as well as in exocrine cells located in other tissues, such as salivary glands, stomach or breast. It has been described to control a set of genes that establish crucial aspects of the machinery for regulated exocytosis in exocrine cells. In the absence of Mist1, pancreatic acinar cells are smaller and disorganized, with loss of apical-basal polarity, reduced stores of secretory proteins, defective calcium-signaling, and aberrant regulated exocytosis [77, 78]. Mist1 may also control genes that help to stabilize the acinar phenotype, since it has been shown that Mist1-deficient acinar cells often co-express ductal markers and are predisposed to ductal metaplasia upon *in vivo* $Kras^{G12D}$ activation [79]. Mist1 target genes in acinar cells include the connexin32 gene Gjb1, which

encodes a gap junction protein [80], Rab3d and Rab26, encoding Ras-like regulators of secretory vesicle formation and transport [81]. The action of the aPTF1 complex complements the function of Mist1 by inducing pancreas-restricted components of the secretory apparatus, in part to accommodate specific secretory proteins and possibly to further enhance the efficiency of intracellular transport and secretory capacity [3].

The function of acinar cells is to synthesize and secrete large amounts of proteins, by which they developed a tight control of the stress responses involved in protein folding. Genes that regulate endoplasmic reticulum homeostasis and control the unfolded protein response (UPR), such as Xbp1 and the Atf/Crb subfamily of transmembrane UPR sensors, have been shown to be highly expressed in acinar cells. Furthermore, these cells are protected from oxidative stress by possessing high levels of the transcription regulators of the cellular response to oxidative stress Nrf1 and Nrf2 [3].

Other transcriptional regulators that may contribute to increase or maintain high level expression of digestive enzyme transcripts in acinar cells include Atf5, Tead2, Klf15, Dbp, Gata4, Foxa2, Foxa3, Spdef, Hnf4a, Nr5a2 and Hnf1a. These are likely regulators of additional aspects of acinar differentiation, although neither their general roles nor specific target genes have been confirmed in the mature exocrine pancreas [3].

I.3.3. The plasticity of acinar cells

Adult pancreatic acinar cells have been shown to display a wide plasticity, being able to give rise to other differentiated types of cells, both pancreatic and non-pancreatic.

The best documented process is the ability of acinar cells to transdifferentiate to ductal-like cells. This has been shown to occur *in vitro* in adherent cultures of human exocrine pancreas [82, 83], in suspension cultures of rat acinar cells [84] and in 3D murine exocrine cell cultures [85]. The work by Means and colleagues using lineage tracing revealed that acinar to ductal conversion is a true transdifferentiation event, mediated by an intermediate step where mature acinar cells dedifferentiate into a nestin-positive precursor population. This phenomenon seems to be regulated by the activation of the EGFR [85] and Notch pathways [86]. Acinar to ductal transdifferentiation has also been demonstrated by lineage tracing *in vivo* both in experimental models of chronic pancreatitis [87, 88] and in mice which develop preneoplasic ductal lesions [89, 90] and/or ductal adenocarcinoma [91].

Acinar cells can also be reprogrammed into endocrine cells. This has been shown, using lineage tracing, in primary cultures of mouse acinar cells treated with EGF and nicotinamide [92]. Recent work, using lectin-based lineage tracing, has shown that rat acinar cells can be converted into beta-like cells *in vitro* and Notch signaling silencing further enhances this phenomenon [93]. A very important study by Zhou et al has revealed that this process can also occur *in vivo*. By transducing acinar cells with 3 defined transcription factors, Ngn3, Pdx1 and MafA, these authors were able to induce the *in vivo* conversion of acinar cells into insulin-producing cells that could revert diabetes in a murine model of the disease [94].

There is also extensive evidence that pancreatic acinar cells can be converted into hepatocytes, which have a common endodermal origin with pancreatic cells. Several studies have shown the appearance of hepatic foci in the pancreas of adult rodents exposed to various experimental treatments [95, 96] and in patients with cancer [97, 98]. It has also been shown to

occur *in vitro* by culturing exocrine pancreatic cells in the presence of dexamethasone [99, 100]. Recently, Wu et al clearly demonstrated, using lineage tracing, that acinar cells directly give rise to hepatocytes via an intermediate state characterized by expression of the Abcg2 transporter and that the phosphoinositide 3-kinase (PI3K) pathway plays a key role in regulating the transdifferentiation process [101].

More remarkably, a study by Bonal and collegues has revealed that acinar cells can lose their epithelial characteristics and transdifferentiate into adipocyte-like cells upon pancreatic-specific inactivation of c-Myc in mice. The authors suggest that a similar epithelial-mesenchymal transition (EMT) process may be occurring in ageing and pancreatitis, conditions which are characterized by exocrine pancreas involution and replacement with adipose tissue [51].

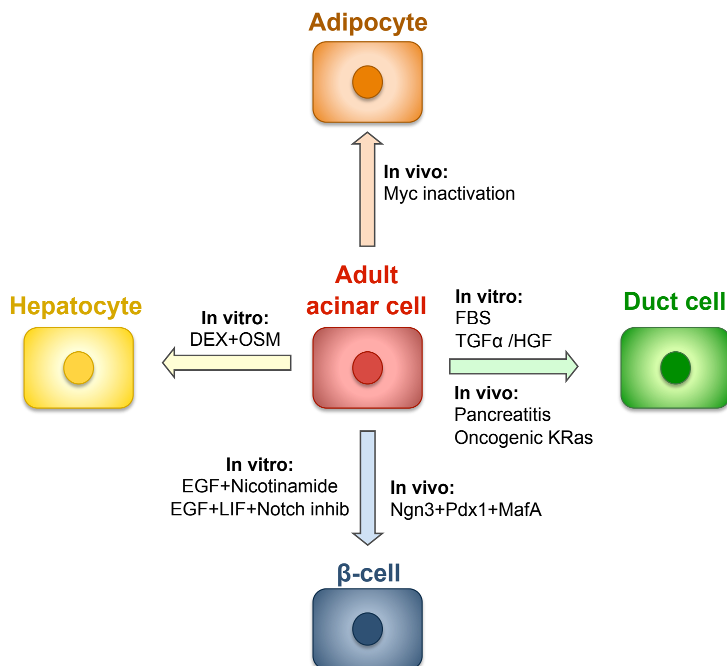


Figure I5. Pancreatic acinar cell plasticity. Pancreatic acinar cells possess the ability to transdifferentiate into other pancreatic cells types, such as ductal and β-cells; and into non-pancreatic cells such as hepatocytes and adipocytes. Adult acinar cells can give rise to duct cells *in vitro* spontaneously, in the case of human adherent cultures [82, 83], and after treatment with the growth factors αTGF and HGF, in mouse 3D cultures [85]. *In vivo*, acinar to ductal transdifferentiation has been seen in mouse models of pancreatitis [87, 88] and in animals where oncogenic Kras was activated in the acinar compartment [89, 91]. Rodent acinar cells can also give rise to β-cells *in vitro*, after treatment with EGF and nicotinamide [92] or with EGF and LIF combined with inhibition of the Notch pathway [93]; and *in vivo*, after induction of expression of the transcription factors Ngn3, Pdx1 and MafA. *In vitro* treatment of murine acinar cells with dexamethasone (DEX) and oncostatin M (OSM) reprograms them into hepatocytes [100, 101]. In a transgenic mouse model, c-myc inactivation in acinar cells resulted in its transdifferentiation into adipocytes [51].

II. Pancreatitis

Pancreatitis is an inflammatory disease of the pancreas that can present as has an acute event or as a chronic manifestation. The major causes of acute and chronic pancreatitis vary in different populations. Gallstones are a major cause of acute pancreatitis with alcohol abuse being associated with both acute and chronic forms of the disease. Acute pancreatitis is thought to initiate within pancreatic acinar cells, subsequent to premature intracellular activation of digestive enzymes, mainly trypsinogen. Repeated attacks of acute pancreatitis have the potential to evolve into chronic disease characterized by fibrosis and loss of pancreatic function [102].

Although often described as two distinct diseases, evidences regarding the genetics of these disorders suggest a continuous range of disease rather than the existence of two separate entities [103].

II.1. Acute pancreatitis

Acute pancreatitis is an acute inflammatory process of the pancreas that frequently involves peri-pancreatic tissues and, less frequently and in its most severe forms, remote organ systems. It is characterized by pancreatic inflammation, edema, necrosis of the acinar cells, and formation of pseudocysts. Eventually, it can give rise to inflammation and injury of non-pancreatic organs. The severity of the disease can vary from mild forms, which are limited to the pancreas, to more severe forms with multisystemic organ failure and death. The overall mortality in patients with acute pancreatitis is 5%, being higher in patients with necrotizing pancreatitis[104, 105].

Acute pancreatitis is believed to originate in acinar cells, which in the early phases of this disease exhibit 3 phenotypic responses: alterations in secretion, the intracellular activation of proteases, and the generation of inflammatory mediators. Under physiologic conditions, the secretion is confined to the pancreatic duct by being limited to the apical membrane that forms the proximal duct lumen. However, this physiologic pattern of secretion can be changed dramatically, early in the course of acute pancreatitis, and may be linked to the pathogenesis of the disease [106].

Acute pancreatitis results from an altered secretion combined with a deregulated activation of the enzyme precursors, the zymogens. This leads to intra-acinar enzyme activation, which causes the auto-digestion of the pancreas and local inflammation. Activation of trypsinogen into active trypsin within acinar cells is thought to be a primary event. It has been proposed that the lysosomal cysteine proteinase cathepsin B (CTSB) plays an essential role in this process [107] . Active trypsin, in turn, activates several other enzymes, such as elastase and phospholipase A2 [104].

Pancreatitis arises when intracellular protective mechanisms to prevent trypsinogen activation or reduce trypsin activity are overwhelmed. Several mechanisms preventing pancreatic auto-digestion by activated trypsin include: the autoproteolysis of trypsin which leads to termination of its activity; the production of serine protease inhibitor Kazal type 1 (SPINK1), which reversibly inhibits activated trypsin; the production of trypsin-activated enzymes that degrade trypsinogen; and the secretion of bicarbonate which is affected by abnormal production of cystic fibrosis transmembrane conductance receptor (CFTR) [103].

Injured acinar cells release cytokines and chemokines that recruit inflammatory cells, such as neutrophils and macrophages, into the

pancreas. The recruitment and activation of inflammatory cells leads to further acinar injury and causes an elevation of various pro-inflammatory mediators, as tumor necrosis factor alpha (TNFα) and interleukins (IL) 1, 2 and 6. In addition, activation of endothelial cells enables the trans-endothelial migration of leucocytes, which release other harmful enzymes. Decreased oxygen delivery to the organ and generation of oxygen-derived free radicals also contribute to injury. Moreover, several signaling pathways have been found to be activated in pancreatitis, being thought to mediate the inflammatory response in the pancreas. These signaling systems include nuclear factor-κB (NF-κB), activator protein-1 (AP-1), mitogen-activated pathway kinase (MAPK), STAT3 and phosphatidylinositol-3 kinase (PI3K). Inhibition of these pathways has been shown to decrease inflammation and to improve the severity of the disease [104, 105].

II.2. Chronic pancreatitis

Chronic pancreatitis is characterized by progressive and irreversible damage to both the exocrine and the endocrine compartments of the pancreas, eventually resulting in severe mal-digestion and diabetes. The histopathologic features of this disease include acinar atrophy, pancreatic fibrosis, chronic inflammation and abnormal ducts. In most patients, abdominal pain is the dominant symptom. Complications ensuing from this disease include pseudocyst formation, pancreatic stones and strictures, biliary and duodenal stenosis, portal hypertension, exocrine insufficiency, malabsorption, and an increased risk of developing pancreatic cancer. The incidence of chronic pancreatitis in industrialized countries ranges from 3.5 to 10 per 100.000 population [103, 108].

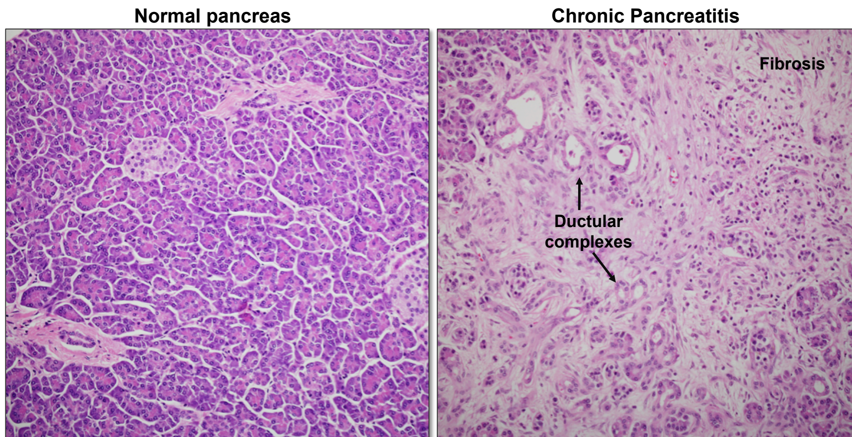


Figure I6. Chronic pancreatitis. Hematoxylin and eosin staining of a normal human pancreas, and of a pancreas with chronic pancreatitis, showing areas with ductular complexes and fibrosis.

Chronic pancreatitis is believed to result from repeated overt or silent episodes of acute pancreatitis, being associated, in the majority of cases, with alcohol abuse [102]. The "necrosis-fibrosis model" of progressive pancreatic injury states that repeated attacks of acute pancreatic necro-inflammation results in increasing residual damage to the pancreas, eventually progressing into an irreversible injury to the gland, characterized by acinar atrophy and fibrosis. Clinical and experimental data support this hypotheses and it is now thought to be applicable, not only to alcoholic pancreatitis, but also to other non-alcohol related forms of the disease in which the clinical course is punctuated with recurrent attacks of necro-inflammation [108]. The molecular mechanisms responsible for pancreatic fibrosis after necro-inflammatory episodes are now better understood after the identification and characterization of the pancreatic stellate cells (PSCs), which play a critical role in the fibrogenic process, as explained in a following chapter.

II.3. The etiology of pancreatitis

II.3.1. Gallstone pancreatitis

Gallstones are the leading cause of acute pancreatitis, accounting for 90% of cases worldwide. It is believed that obstruction of the pancreatic duct is a critical event in gallstone pancreatitis. At the cellular level, the obstruction of the pancreatic duct would impede acinar exocytosis, leading to intra-acinar activation of trypsinogen. The consequent cascade of digestive enzyme activation within the acinar cell leads to autodigestive injury to the gland [102, 103].

II.3.2. Alcoholic pancreatitis

Alcohol abuse is the major cause of chronic pancreatitis, being responsible for 70% of cases of chronic pancreatitis. It is also cited as the second leading cause for acute pancreatitis, although it is controversial whether acute alcoholic pancreatitis can arise in the absence of a chronic injury to the pancreas [103].

Alcohol consumption affects the pancreas through several mechanisms. It is known that chronic ethanol administration in animal models decreases pancreatic secretion, a phenomenon related to disturbances in exocytosis, with subsequent accumulation of enzymes within the cell. In addition, it leads to a decrease in the stability of zymogen granule and lysosomal membranes. The pancreas is capable of metabolizing ethanol via the oxidative pathway - converting it to acetaldehyde - and the non-oxidative pathway - converting it to fatty acid ethyl esters (FAEE). Both these metabolites have been shown to cause toxic effects in the pancreas. Furthermore, a byproduct of ethanol oxidation is the formation of reactive

oxygen species, which are highly reactive compounds that are potentially harmful to the cell [102].

Although it is now clear that alcohol consumption predisposes the pancreas to auto-digestion and necro-inflammation, only a minority of heavy drinkers develops pancreatitis, indicating that an additional insult or second hit is required. Although many studies have been conducted over the past years, the factors that confer susceptibility to alcoholic pancreatitis remain unknown [108].

II.3.3. Hereditary pancreatitis

For many years it has been recognized that pancreatitis clusters in selected families, suggesting a hereditary form of the disease in these patients. In 10-30% of patients with chronic pancreatitis, no apparent underlying cause can be identified and there is no familial aggregation of the disease, being classified as idiopathic pancreatitis. Nevertheless, as knowledge in the field progresses, many of the so-called idiopathic cases are being found to have a genetic component [108].

Using linkage analysis, a gene for hereditary pancreatitis has been identified which is located in chromosome 7q35, corresponding to the cationic trypsinogen gene [109]. Three different trypsinogen isoforms have been described according to their electrophoretic mobility, cationic trypsinogen (PRSS1), anionic trypsinogen (PRSS2) and mesotrypsinogen (PRSS3). The first genetic association with pancreatitis was described within the *PRSS1* gene (mutation R122H). This mutation leads to a gain of function characterized by enhanced trypsinogen auto-activation and inhibition of autolysis of the enzyme. R122H appears as the most common mutation in *PRSS1* but several other mutations have been identified in families with hereditary pancreatitis [108]. The relevance of R122H mutation as a

pathogenic mediator of pancreatitis has been supported by the development of the corresponding transgenic mouse. These animals displayed early onset acinar injury, inflammatory cell infiltration, enhanced response to caerulein-induced pancreatitis and developed fibrosis and acinar cell dedifferentiation with ageing [110].

Mutations in the anionic trypsinogen gene (*PRSS2*) have been shown to be protective against pancreatitis. The G191R mutation was found in 1.3% of patients with chronic pancreatitis and in 3.4% of controls. *In vitro* studies have shown that this variant contains a novel tryptic cleavage site rendering it more vulnerable to catalytic autodigestion [111].

Rosendahl et al. have identified two mutations in the Chymotrypsin C gene as significantly over-represented in a cohort of patients with idiopathic and hereditary chronic pancreatitis. *In vitro* functional assessment of the two mutations showed loss of function due to impaired activity and/or reduced secretion of the protein [112].

The serine protease inhibitor Kazal type 1 (*SPINK1*) is a potent inhibitor of trypsin activity within the pancreas. Mutations in *SPINK1* have been found to be associated with pediatric [113] and familial pancreatitis [114]. The first mutation described in this gene was the N43S mutation whose pathophysiological role remains unknown as recombinant SPINK1^{N43S} has normal trypsin inhibitor capacity. Other mutations leading to the secretion of a truncated protein or to the absence of secretion have also been reported [102]. Two mouse models have recently corroborated the role of *SPINK1* in the pancreas. Transgenic overexpression of SPINK1 protects against experimental pancreatitis whereas targeted deletion of the murine homologue of *SPINK1* leads to degeneration of acinar cells and death within 2 weeks after birth [115, 116].

CFTR is expressed in the proximal ductal system of the pancreas and it regulates bicarbonate and chloride secretion. Abnormal CFTR function as a result of mutations has been associated with cystic fibrosis and a minority of these patients suffers recurrent attacks of acute pancreatitis. Interestingly, patients with idiopathic chronic pancreatitis have up to 6-fold increased likelihood of carrying a *CFTR* mutation in heterozygosity, as compared to the general population. According to studies where the entire *CFTR* gene has been sequenced in patients with idiopathic chronic pancreatitis, 25–30% of patients carry at least one mutation. Thus, idiopathic chronic pancreatitis may represent in fact an ”atypical” cystic fibrosis [108]. It is noteworthy that the combination of a *CFTR* and a *SPINK1* or *PRSS1* mutation has also been found to be associated with alcoholic pancreatitis [102].

II.3.4. Autoimmune pancreatitis

Autoimmune pancreatitis is a rare, non-alcohol related, form of chronic pancreatitis that appears is association with other autoimmune diseases. It is characterized by the presence of increased serum γ-globulin levels and IgG4 autoantibodies, and pancreatic fibrosis with lymphocytic infiltration. The pathogenesis of this disease remains unknown, but it is postulated that it arises from an aberrant expression of the major histocompatibility complex HLA-DR in ductal and acinar cells that leads to the presentation of auto-antigens to lymphocytes [108].

II.3.5. Tropical pancreatitis

Tropical pancreatitis is a form of chronic pancreatitis described in tropical countries such as India, Sub-Saharan Africa and the West Indies, manifesting predominantly in patients under the age of 25 years. The

disease is characterized by the presence of abundant calcifications. Several etiological factors have been proposed such as malnutrition, cyanogenic glycosides, viral and parasitic infections, autoimmune mechanisms, and oxidative stress. However, there is little evidence supporting a role for these factors in the pathogenesis of tropical pancreatitis. Regarding genetic factors, there have been reported positive associations of tropical pancreatitis with mutations in the genes coding for SPINK1, Cathepsin B and with mutations in the Chymotrypsin C gene [102]. Whether tropical pancreatitis truly constitutes a distinct pathogenic entity is a matter of current debate.

II.4. Pancreatic stellate cells: the role in pancreatitis

Pancreatic stellate cells (PSCs) are resident cells of the normal pancreas, constituting 4% of all pancreatic cells [117]. PSCs show mainly a peri-acinar distribution and are in a quiescent state. These cells can be identified by the presence of vitamin A–containing lipid droplets in the cytoplasm and expression of the intermediate filament proteins, desmin and glial fibrillary acidic protein (GFAP) [118]. PSCs share many morphological and functional characteristics with hepatic stellate cells (HSCs), whose central role in liver fibrosis is well established. Normally, PSCs contain substantially lower vitamin A levels than their hepatic counterpart [119]. Other differences between the two stellate cell types, involve expression of genes associated with ECM production and turnover, cell adhesion, cell communication and cytoskeleton [120].

In response to pancreatic injury or inflammation, quiescent PSCs undergo activation to become myofibroblast-like cells, which express α-SMA. Upon activation, PSCs lose lipid droplets, proliferate, migrate, produce ECM components, and secrete pro-inflammatory cytokines and chemokines.

Cytokines and growth factors produced by acinar cells, inflammatory cells, platelets, ductal cells, endothelial cells, cancer cells, and PSCs by themselves could activate PSCs, and induce cellular responses trought paracrine and autocrine mechanisms. Chemokines produced by PSCs contribute to the recruitment of inflammatory cells to the inflamed pancreas. PSCs also produce matrix metalloproteinases (MMPs) and their inhibitors, being involved in the maintenance of normal tissue architecture by regulating ECM turnover. It is also thought that PSCs play a “macrophage-like” role in the pancreas, contributing to organ restitution and homeostasis by engulfing pancreatic acinar cells undergoing apoptosis and necrosis [117].

Activated PSCs can have 2 fates. If the inflammation and injury are limited, as in the case of an acute episode of pancreatitis, PSCs might undergo apoptosis or revert to quiescence. If the inflammation and injury are sustained or repeated, PSC activation is perpetuated, leading to the development of pancreatic fibrosis, as observed in chronic pancreatitis. Thus, pancreatic fibrosis results from pathologic changes of ECM composition that result from persistent PSC activation due to the repeated or sustained injury and inflammation of the pancreas, in accordance with the “necrosis-fibrosis model” [108, 117].

In addition to their roles in chronic pancreatitis, it is increasingly recognized that PSCs contribute to the progression of pancreatic cancer. Pancreatic cancer cells recruit PSCs to their immediate vicinity to establish a supportive and permissive microenvironment. On the other hand, PSCs can also promote tumoral cell proliferation and migration [118].

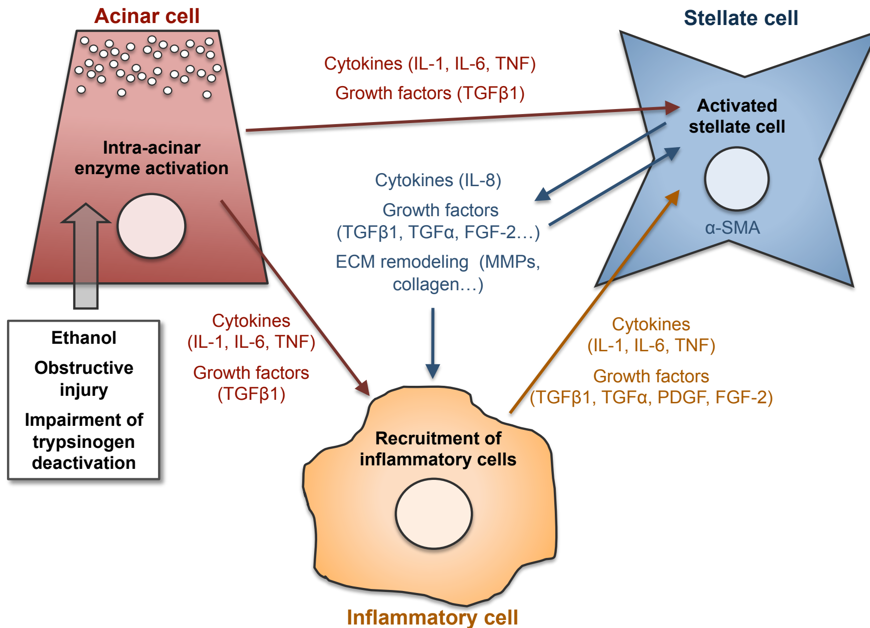


Figure I7. Interaction between pancreatic acinar, stellate, and inflammatory cells during the pathogenesis of chronic pancreatitis. Ethanol consumption, obstruction of the pancreatic duct or an impairment of the mechanisms that prevent trypsynogen activation (i.e. mutations in genes involved in this pathway) can lead to an intra-acinar activation of trypsin and consequent activation of other digestive enzymes. The injured acinar cell secretes pro-inflammatory cytokines and growth factors, which activate pancreatic stellate cells (PSCs) and, at the same time, recruit inflammatory cells into the affected area, which can also secrete factors that activate PSCs. Activated PSCs express α-smooth muscle actin (α-SMA), secrete cytokines and growth factors which further contribute to the recruitment of inflammatory cells, and produce extracellular matrix (ECM) proteins, such as collagen and metaloproteinases (MMPs), contributing to the formation of the fibrosis observed in the pathogenesis of chronic pancreatitis.

II.5. Experimental models of pancreatitis

Several animal models have been developed to study acute and chronic pancreatitis. In fact, only animal models provide the ability to reveal the sequence of initiating molecular steps resulting in pancreatitis.

Rodent animal models for pancreatitis can be non-invasive or non-surgical such as chronic ingestion of ethanol and administration of supramaximal stimulatory doses of caerulein (see below) or a toxic substance. Interestingly, long-term ethanol feeding alone is unable to induce acute or chronic pancreatitis in rodents. The second group includes the invasive or surgical models, such as manipulation of the pancreatic duct or infusion of some toxic substances into the pancreatic duct [121].

Nevertheless, none of the models that have been extensively used in the literature are fully representative of human pancreatitis and indeed the majority of them fails to recapitulate the complete spectrum of the human disease. Thus, considerable caution should be used when applying the results from experimental animal models to design therapies for humans[105].

II.5.1. Acinar to ductal transdifferentiation in experimental models of pancreatitis

Acute pancreatitis has been widely studied in rodents by using caerulein, a cholecystokinin agonist that regulates secretion of exocrine enzymes. Caerulein treatment induces pancreatic edema and loss of acinar cells which are transiently replaced by a duct-like epithelium, reminiscent of human pancreatitis. When the insult is ceased, the metaplastic epithelium undergoes regeneration, differentiating back to the acinar phenotype [88, 122]. A similar phenomenon is observed in studies where pancreatic regeneration is induced by partial pancreatectomy [87]. Lineage tracing experiments have shown that mature acinar cells have the ability to transdifferentiate, giving rise to the intermediate ductal metaplastic epithelium present in pancreatitis [87, 88, 122].

Several studies have shown that caerulein injury and subsequent regeneration seem to recapitulate the steps involved in pancreatic

embryonic development since both Pdx1 and the Notch pathway are reactivated [123, 124]. Interestingly, in a rat model of pancreatic duct ligation in which acini become metaplastic and transform into tubular duct-like structures, the newly formed ductal epithelium was also found to reactivate the expression of Pdx1 and to present activated Notch pathway [86]. Results obtained through the inhibition of Notch signaling by chemical and genetic methods revealed that this pathway is required for exocrine pancreas regeneration and suggested a modulatory function of Notch on the activity of β-catenin, providing evidence for a role of both Notch and Wnt pathways in the regulation of the maturation process of acinar cells [124]. Furthermore, Hedgehog signaling has also been found to be activated during pancreatitis. Mice deficient in Hedgehog activity develop persistent ductal metaplastic lesions being unable to regenerate new mature acinar cells and expressing markers of pancreatic progenitor cells [122].

III. Pancreatic Ductal Adenocarcinoma

Pancreatic Ductal Adenocarcinoma (PDAC) is the most common neoplasm of the pancreas, accounting for more that 85% of pancreatic tumor cases[125]. PDAC represents a remarkable problem for western society, being the fourth cause of cancer related deaths in the United States[126] and the fifth in Europe [127] and it was estimated to have caused around 266.000 deaths worldwide during 2008 [127]. The astonishing mortality observed in this disease is related to the fact that it is one of the tumors with a worst prognosis, presenting a median survival of < 6 months and a 5-year survival rate of <5% [125]. Despite the tremendous scientific efforts to unravel the molecular basis of PDAC and the recent development of genetic mouse models of the disease, the survival rates have remained virtually unchanged over the last 50 years and much remains to be done in order to have a profound knowledge of the mechanisms that regulate the initiation, progression and metastasis of this carcinoma.

III.1. PDAC pathology

PDAC arises more frequently in the head, neck or uncinate process of the pancreas (60-70% of cases), whereas presentations in the body (5-10%) or tail (10-15%) of the gland are less common. Patients often suffer from dull, deep upper abdominal pain and present with asthenia, anorexia and severe weight loss. The tumor commonly causes obstructive cholestasis and the obstruction of the main pancreatic duct can lead to pancreatitis and eventually to dysglycemia [128].PDAC has a high propensity for local invasion and distant metastases. Perineural, vascular and lymphatic invasion are commonly observed in resected tumor specimens and metastasis are more common in liver, peritoneum, lung and lymph nodes [129, 130].

Most cancers of the pancreas are infiltrating ductal adenocarcinomas, but there are some less common tumors including those with colloid, adenosquamous, or sarcomatoid histology. At the microscopic level, PDAC is composed of an infiltrating gland-forming neoplastic epithelium, with an intense desmoplastic reaction [131]. A mixed population of fibroblastic, endothelial and inflammatory cells constitutes the dense stroma present in PDAC. Pancreatic stellate cells are an important subpopulation of the stroma and play a critical role in the desmoplastic response. The stroma, usually considered a mechanical barrier, in fact constitutes a dynamic compartment that is strongly involved in the process of tumor formation, progression, invasion and metastasis [128].

III.1.1. Treatment of PDAC

Most patients with PDAC present with a locally advanced unresectable tumor due to local vascular invasion, or metastatic disease at diagnosis and it is estimated that only 10-15% of patients present with early-stage disease that permits surgery with curative intent. Post-operative adjuvant treatment is the standard of care for patients with early-stage pancreatic cancer who undergo radical surgery. However, no universal consensus exists as to the type of adjuvant therapy. Cytotoxic chemotherapeutic agents, such as gemcitabine or 5-fluorouracil without radiation, are the most common treatments outside North America, while chemo-radiation plus systemic chemotherapy is still widely used in the US. The median survival for patients undergoing surgery followed by adjuvant treatment is approximately 23 months and 5-year survival is of 20%[130, 132].

Approximately 50% of patients with pancreatic carcinoma present with advanced-stage disease that has extended beyond the pancreas and regional lymph nodes with metastases to the liver or other visceral organs.

The median survival for these patients is around 6 months [130]. For more than a decade, the treatment of choice for patients with metastatic disease has been gemcitabine. Multiple new agents with diverse mechanisms of action have been tested, in randomized clinical trials, in combination with gemcitabine, with no improvement in outcome. Erlotinib, a small-molecule inhibitor of epidermal growth factor receptor, is the only agent that, in combination with gemcitabine, has shown a small but significant improvement in median survival (15 days)[128].

Overall, the strategy for PDAC treatment has been largely unsuccessful and new approaches are required in the areas of clinical, translational and basic research. A better understanding of the signaling pathways and underlying mechanisms that play a critical role in PDAC development could help in the development of new and more-active targeted agents that could overcome the intrinsic resistance of pancreatic cancer cells to most current treatments[130].

III.2. PDAC epidemiology

Pancreatic cancer etiology, like many other complex diseases, is associated with both environmental and genetic components.

III.2.1. Environmental Factors

The incidence of pancreatic cancer varies greatly with latitude, with rates being higher in cold northern countries and lower in countries close to equator. One proposed reason for this divergence is that cancer risk increases with decreased levels of vitamin D, which are related to sunlight and ultraviolet exposure [132].

PDAC is predominantly a disease of the elderly and the median age at diagnosis of the disease is around 70 years. The lifetime cumulative probability of developing pancreatic cancer is about 1% for men, being slightly less for women. There are also differences between races, with the frequency of pancreatic cancer being higher in the black population than in white people and the lowest rates are found for Asian populations [132].

Smoking is the best established risk factor for PDAC, causing approximately a 2-fold increase in the risk of pancreatic cancer in smokers compared to non-smokers and being estimated to be cause approximately 20-30% of all cases [133]. Alcohol consumption does not seem to be a strong risk factor [134] although it may do so through its contribution to chronic pancreatitis. Furthermore, no strong or consistent association has been found between diet, physical activity or drug use and pancreas cancer risk [132].

III.2.2. Association with other diseases

Chronic pancreatitis has been shown to be a risk factor for pancreas cancer[135-137]. In a large retrospective cohort study, Lowenfels et al. reported that patients who had been diagnosed with chronic pancreatitis for at least 5 years prior to the diagnosis of PDAC had an increased risk of developing cancer of 14-fold [136]. The association between chronic pancreatitis and PDAC is even stronger among patients with an autossomal dominant hereditary form of this disease [138, 139] (see below).

While it is clear that diabetes diagnosis often shortly foreruns PDAC diagnosis, there is also strong evidence that long-term history of the disease can contribute to PDAC. Several studies have demonstrated that patients with type 2 diabetes have an increased risk of developing PDAC that can be up to 50% in patients with long term disease [140 141].

Obesity has also been shown to be a risk factor for several cancers, including PDAC [142] and Li et al. have shown that overweight during early adulthood is associated with a greater risk of pancreatic cancer and a younger age of disease onset [143].

III.2.3. Genetic susceptibility

Genetic factors play a role in the etiology of PDAC, as shown by the familial aggregation of the disease, with around 10% of patients reporting a family history of the disease. First-degree relatives of PDAC cases have an estimated risk of 2- to 4-fold, and this risk increases up to 57-fold when 3 or more relatives are affected [144].

Familial PDAC aggregation has been linked with germline mutations in genes that cause familial cancer syndromes and where pancreatic cancer is part of the spectrum of the disease. In this category are included the genes *BRCA1/2* (Familial breast–ovarian cancer), *p16/INK4A/CDKN2A* (Familial atypical mole-multiple melanoma, FAMMM), *STK11/LKB1* (Peutz-Jeghers syndrome), *TP53* (Li-Fraumeni syndrome), *APC* (familial adenomatous polyposis), as well as the DNA mismatch repair genes *MLH1* and *MSH2* (Lynch syndrome/Human non-polyposis colorectal cancer)[145].

Nevertheless, these germline mutations are estimated to account only for around 20% of familial pancreas cancer cases [125]. One study found strong evidence of linkage of familial pancreatic cancer to a common locus at 4q32.34 involving *PALLD* [146], but this genetic alteration has not been identified so far in any other study.

As referred above, chronic pancreatitis can increase the risk of developing PDAC and patients with hereditary pancreatitis presenting mutations in the

cationic trypsinogen gene (*PRSS1*) have been shown to have 53-87-fold increased incidence of PDAC [138, 139].

Another link has been found between the cystic fibrosis gene (*CFTR*) and PDAC, as one group has shown that *CFTR* mutations are associated with early onset of the disease [147, 148]. Nevertheless, previous studies had found no association between *CFTR* mutations and PDAC [149, 150]. Thus, it is likely that the type of CFTR mutation influences the risk of PDAC development among carriers.

Common genetic variants or polymorphisms are likely to play an important role in both familial and sporadic forms of PDAC, either individually or in interaction with environmental factors. The most important genetic associations found in sporadic pancreatic cancer include genes involved in carcinogen metabolism, DNA repair, inflammation, carbon metabolism and mitochondrial genes. Nevertheless, few polymorphisms have been identified and most of the findings have not been replicated in independent studies. More comprehensive and agnostic approaches, such as genome-wide association studies (GWAS) are likely to be more informative than candidate gene studies 144. In fact, a very recent GWAS study identified new pancreatic cancer susceptibility loci on chromosomes 13q22.1 (corresponding to an intragenic region between two genes of the family of Kruppel-like transcription factors, KLF5 and KLF12), 1q32.1 (containing the NR5A2 gene), and 5p15.33 (containing the locus that codes for the telomerase gene (TERT) and for the CLPTM1L gene) 151. A previous GWAS study had already shown that variants in the ABO locus are also associated with susceptibility to pancreatic cancer [152].

III.3. Molecular genetics and PDAC progression model

The most widely accepted model of PDAC progression states that this tumor arises from histologically well-defined precursor lesions in the small ducts of the pancreas, through the accumulation of multi-step genetic alterations [131, 153].

This model is based on the study of non-invasive lesions named Pancreatic Intraepithelial Neoplasia [154]. PanINs are microscopic lesions in the small (<5 mm) pancreatic ducts. These lesions can be papillary or flat, and they are composed of columnar to cuboidal cells with varying amounts of mucin. Depending on the degree of cytological and architectural atypia they are subclassified into PanIN-1, PanIN-2, and PanIN-3 lesions. PanINs are often found in the pancreatic parenchyma adjacent to infiltrating adenocarcinomas, and several case reports have documented patients with PanINs who later developed pancreatic cancer [131].

The major evidence that led to the development of the linear progression model for PDAC is the observation that PanINs harbor many of the genetic alterations that are found in invasive pancreatic cancer. The main genetic abnormalities that would give rise to PDAC, in the chronologic order proposed by the model, are: *KRAS* mutations and loss of *p16/INK4A/CDKN2A*, *TP53* and *SMAD4/DPC4* [131, 153].

III.3.1. The *KRAS* oncogene

KRAS mutations are thought to be the earliest genetic events to occur during PDAC progression, being found in 90%–95% of cancer cases and in approximately 36%, 44%, and 87% of cancer-associated PanIN-1A, PanIN-1B, and PanIN-2/3 lesions [131].

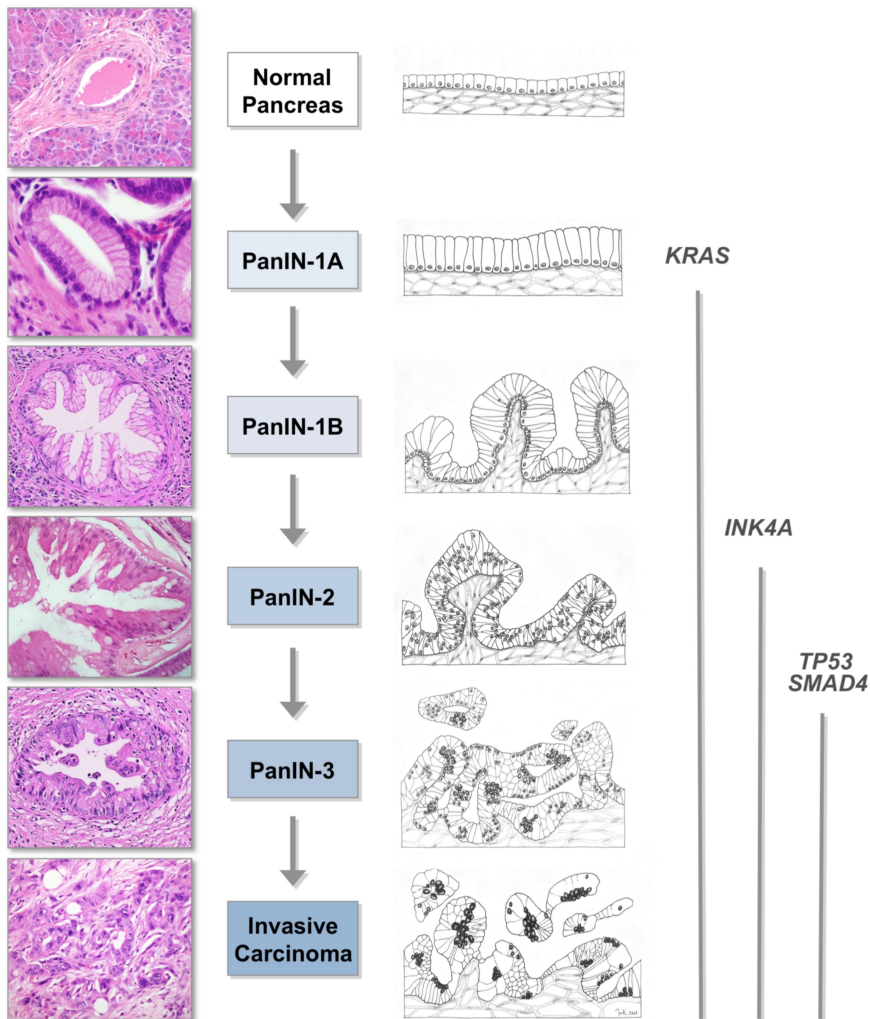


Figure I8. Pancreatic ductal adenocarcinoma (PDAC) linear progression model. PDAC is thought to derive from precursor lesions designed pancreatic intraepithelial neoplasias (PanINs). PanINs represent progressive stages of neoplastic growth and are graded from stages I to II, with the earliest stage being characterized by the appearance of a columnar, mucinous epithelium, and with increasing disorganization and nuclear atypia through stages II and II. The same genetic alterations documented in PDAC also occur in PanINs, in what seems to be a temporal sequence. *KRAS* activating mutations are thought to be the first event to occur in the early stage PanINs, being followed by loss of *INK4A*, and later on, by loss of *TP53* and *SMAD4*. *Illustrations were adapted from Real, 2003* [155].

The high frequency of *KRAS* gene mutations in human PanINs supports its role as an initiating event for pancreatic cancer formation and this has been reiterated through the use of murine experimental models. Several mouse models engineered to express an oncogenic form of KRas develop the whole spectrum of PanIN lesions found in association with PDAC [89-91, 156] (discussed in detail in the next section). In addition to its role in pancreatic cancer initiation, constitutive RAS signaling appears to be required for pancreatic cancer maintenance as well [157].

KRAS belongs to a group of small guanidine triphosphate (GTP) binding proteins which oscillate between an active (GTP-bound) and an inactive (GDP-bound) state [158]. PDAC harbors *KRAS* activating point mutations located in codon 12 that inhibit the protein's ability to hydrolyze GTP, resulting in a constitutively activated molecule, independent of growth factor stimulation [125]. Activated KRAS engages multiple effector pathways that promote cell proliferation, survival, migration and differentiation. The best established RAS downstream targets are the RAF-mitogen activated protein kinase (MAPK), the phosphoinositide-3-kinase (PI3K) and RAL-GDS pathways [158].

The importance of the RAF-MAPK pathway in cancer has emerged with the identification of activating mutations in B-RAF in many malignancies. Although rare in PDAC, *B-RAF* mutations are present in one-third of pancreatic cancers with wild-type *KRAS* [159], resulting in the activation of RAF-MAPK signaling even in the absence of *KRAS* mutations. Furthermore, abrogation of RAF-MAPK signaling with small molecule inhibitors, or with antisense inhibition of an essential transducer of RAS signaling to RAF, results in growth inhibition of pancreatic cancer xenografts, evidencing the critical role for RAF-MAPK signaling in pancreatic carcinogenesis[125].

The PI3K-AKT pathway is an essential cell survival pathway, with diverse roles in tumorigenesis across multiple solid malignancies. The PI3K pathway is constitutively activated in most pancreatic cancers, and targeting this pathway with small molecule inhibitors or genetic strategies results in growth inhibition *in vitro* and *in vivo* [131]. Although RAS certainly contributes to PI3K-AKT signaling in pancreatic cancer, independent genomic events can also activate this pathway, including amplification of the AKT2 gene in 10-20% of PDAC cases [160, 161]. By contrast, PIK3CA mutations, which are common in other tumors, are rare in classical PDAC [162].

III.3.2. p16 and the *INK/ARF* locus

The gene coding for *p16/INK4A/CDKN2A* is the most commonly inactivated tumor suppressor gene in pancreatic cancers. Loss of p16 function occurs in approximately 90-100% of pancreatic cancers and occurs through several different mechanisms, including homozygous deletion (40%), intragenic mutation with loss of the second allele (40%), and epigenetic silencing by promoter methylation (10%–15%) [131]. Several studies have shown that loss of p16 function occurs most frequently, but not exclusively, in higher grade lesions PanIN-2 and PanIN-3 [163-165] and thus it was proposed that this event would occur after *KRAS* mutation in the PanIN-PDAC progression model [153].

INK4A belongs to the cyclin-dependent kinase (CDK) inhibitor family and the protein inhibits cell cycle progression through the G1-S checkpoint, by inhibiting CDK4/6-mediated phosphorylation of RB. The 9p21 locus encodes not only for *INK4A* but also for another tumor suppressor that uses a different exon 1, in an alternative reading frame: *ARF* (p19 in mouse, and p14 in humans). This protein has been shown to stabilize p53 by inhibiting MDM2-dependent proteolysis [166]. Moreover, these two tumor suppressors have been found to be upregulated in oncogenically stressed cells and to be

responsible for the cell cycle arrest imposed on these cells, through a mechanism called senescence[167], which will be further discussed in a following chapter. Given the physical juxtaposition and frequent homozygous deletion of 9p21 in 40% of PDAC cases, many pancreatic tumors sustain loss of both *INK4A* and *ARF* tumor suppressor pathways. Nevertheless, mutations that selectively target *ARF* are rare and many germline and sporadic mutations exclusive for *INK4A* have been identified, supporting the notion that this gene contributes most to PDAC [125].

III.3.3. The *TP53* tumor suppressor

Inactivation of the *TP53* gene on chromosome 17p is present in approximately 50%–75% of pancreatic cancers, and almost always occurs via intragenic mutation combined with loss of the second allele. *TP53* loss occurs almost exclusively in the most advanced lesions, PanIN-3 or carcinoma in situ [131, 153].

p53 is a major regulator of responses to stress. Under normal conditions is functionally inactive due to its rapid degradation by the ubiquitin ligase MDM2. Upon stress, including DNA damage or oncogenic signaling, MDM2-driven degradation is halted and p53 accumulates, gaining full competence in transcriptional activation. p53 targets include a number of cell cycle inhibitors and pro-apoptotic proteins, which results in cell-cycle arrest, senescence or apoptosis. Loss of p53 function allows cells to survive and divide despite the presence of damaged DNA, leading to the accumulation of additional genetic abnormalities [168].

III.3.4. *SMAD4* and TGF-β signaling

The *SMAD4/DPC4* (deleted in pancreatic carcinoma 4) gene, located on chromosome 18q21, is inactivated in approximately 55% of pancreatic

cancers, either by homozygous deletion (30%) or by intragenic mutations and loss of the second allele (25%). As with *TP53*, this gene is usually inactivated in PanIN-3 lesions, by which these two events were considered to occur late, in the linear progression model for PDAC [131, 153].

SMAD4 is a transcriptional regulator that plays a critical role in the transforming growth factor-β (TGF-β) pathway. TGF-β is a cytokine that signals through serine/threonine kinase receptors known as the type I and type II receptors. On binding to TGF-β, the type II receptors phosphorylate and activate the type I receptors that then propagate the signal by phosphorylating Smad transcription factors (i.e. Smad2 and 3), which shuttle to the nucleus where they form a complex with Smad4, activating a variety of downstream targets [169].

TGF-β is a potent inhibitor of epithelial cell growth and survival through modulation of expression of cell cycle regulators and activation of apoptosis, although these effects are highly dependent on cellular context[170]. On the other hand, TGF-β can enhance the malignant growth of established epithelial tumors, promoting tumor cell proliferation, migration, and epithelial-mesenchymal transition (EMT)[171]. Therefore, TGF-β signaling can have a dual context-dependent function, inhibiting carcinoma initiation and promoting the high-grade advancement and dissemination of established tumors. Due to this dual function of TGF-β, the impact of loss of *SMAD4* in PDAC prognosis has not yet been clearly established. In fact different studies have reached opposite conclusions regarding the status of *SMAD4* and survival [172, 173].

III.3.5. Other genetic alterations

Somatic mutations in genes responsible for inherited disorders associated with pancreatic cancer have also been implicated in the development of

sporadic forms of this disease, such as inactivation of the serine-threonine kinase *LKB1/STK11*. The TGF-β/activin signaling pathway receptors such as *TGFBR1*, *TGFBR2*, and *ACVR1B* have also been found to be inactivated in a small proportion of pancreatic cancers [131].

In addition to classic oncogenes and tumor suppressors, several genome maintenance genes are also inactivated in sporadic pancreatic cancer. These genes do not directly influence cell growth and proliferation, but rather prevent the accumulation of DNA damage and maintain genomic fidelity, such as the DNA mismatch repair genes *hMLH1* and *hMSH2*, *BRCA2,* and related Fanconi anemia (*FANC*) genes [131].

III.3.6. Telomere shortening in PDAC

Van Heek and colleagues were the first to show that telomere erosion is one of the earliest demonstrable genetic aberrations in pancreatic cancer, with >90% PanIN lesions, including PanIN-1, demonstrating marked shortening of telomeres, as compared with normal ductal epithelium [174].

Human telomeres consist of repetitive DNA sequences that associate with a series of telomere binding proteins, the shelterins, which confer stability to chromosomes during cell division. Loss of telomeres renders chromosome ends highly recombinogenic, with the formation of end-to-end fusions that result in chromosome breakage during anaphase, creating regions of amplifications and deletions and the acquisition of chromosomal rearrangements in the daughter cell genome [175].

It has been shown that cells with critically short telomeres activate a p53-dependent DNA damage response and thus, immortalization and transformation of these cells would require the deactivation of this checkpoint. Nevertheless, p53 is lost only in 50-75% of PDAC cases which

raises the possibility that other p53 pathway components involved in the telomere-induced checkpoint responses are neutralized in a subset of these neoplasms. Alternatively, the loss of p53-independent responses in some tumors could obviate the need to inactivate this pathway. These observations underscore the need to specifically correlate telomere length, p53 status, and the onset of genomic instability in PanINs, and to develop pancreatic cancer models with telomere dysfunction [125].

III.3.7. The alternative model

The linear progression model proposed by Hruban et al. [153] has recently been questioned by our group given a number of limitations that may not have been appropriately taken into account by the scientific community, leading to a rapid establishment of a "dogma" model. These limitations have been described elsewhere [176]. The existing phylogenetic evidence supporting PanIN-1 to PDAC progression, and the prevalence of genetic changes, do not allow to conclude about the sequence in which a given genetic alteration occurs, and could actually be interpreted differently. We have proposed instead, an alternative model where *KRAS* mutations might favor the appearance of dysplasia and tumor progression, only when occurring in cells harboring loss of heterozygosity (LOH) in a crucial tumor suppressor gene, such as *INK4A* or *TP53*. On the other hand, *KRAS* mutations occurring in normal cells might lead to growth arrest by activation of senescence. This would mean that only PanIN-2/3 would progress into PDAC while PanIN-1, presenting only *KRAS* mutations, would undergo cell cycle arrest [176]. The discussion is, thus, whether the readout of a *KRAS* mutation is dependent on the genetic context in which it takes place, a concept that is by no means new, when discussing the genetic

evidence for the role of RAS genes in other tumor types (i.e. KRAS and APC in colorectal cancer [177]).

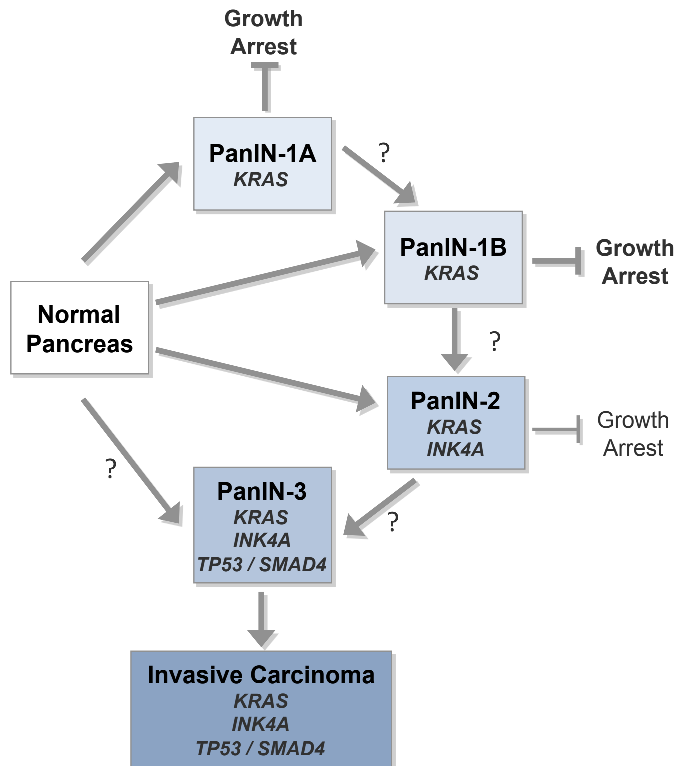


Figure I9. An alternative model of PDAC progression. PanIN-1 lesions may follow a progression route different from that of PanIN-2/3 lesions. In the first case, *KRAS* activating mutations occurring in normal cells, would result in the generation of oncogenic stress, leading to the activation of growth arrest by tumor suppressor genes such as *INK4A* and *TP53*. By contrast, *KRAS* mutations occurring in cells in which LOH at a tumor suppressor loci has already occurred would allow the cell cycle progression of these cells, which eventually accumulate further mutations, giving rise to PDAC. *Adapted from Real et al., 2008* [176].

The proposed model is supported by results obtained using global transcriptome analysis of human PanINs, showing that the expression profiles of PanIN-1 lesions were similar to those of normal ducts, while PanIN-2 and 3 cluster together with PDAC samples [178].

Moreover, in the last few years it has been firmly established using *in vitro* and *in vivo* studies the existence of oncogene-induced senescence, which would function as a protective mechanism against tumor progression in cells that are subjected to oncogenic stress [179-182]. In fact, in a mouse model of PDAC expressing oncogenic *Kras* in the pancreas, it has been shown that PanINs were positive for senescence markers while PDAC was negative [183].

III.4. Genetic approaches to model PDAC in mice

III.4.1. Kras and the cell of origin of PDAC

Due to the morphological similarities of PDAC and PanINs with ductal cells, it was historically believed that PDAC would develop from normal pancreatic duct cells. Nevertheless, the findings from recent development of several PDAC genetic mouse models call for a revision of this concept.

Sandgren et al. provided the first evidences that PDAC could arise from acinar cells: transgenic mice in which c-myc is targeted to pancreatic acinar cells in the embryo develop mixed acinar and ductal pancreatic adenocarcinomas [184]. In another model developed by the same research group, transgenic expression of TGFα specifically in acinar cells led to the development of acinar to ductal metaplasia and PDAC [185].

More recent PDAC mouse models have modeled more closely the human disease using oncogenic KRas as an initiating event. Hingorani and collaborators showed the ability of mutant KRas to drive the formation of PDAC by using a Cre-inducible conditional allele lox-stop-lox (LSL)-$KRas^{G12D}$ targeted to the endogenous locus, whose expression was selectively activated in the pancreatic progenitors around E9 by Cre recombinase under the control of *Pdx1* regulatory sequences. Similar results are obtained using Cre driven from the endogenous *Ptf1a* locus. Both models

recapitulate the stepwise progression of PanINs observed in the human disease and developed invasive carcinoma over the course of one year [156].

Other genetic tools have been used to test the ability of mutant Kras to induce neoplastic lesions when activated in specific subsets of pancreatic cells. One of the first intents was to express oncogenic Kras in ductal cells under the control of the ductal-specific keratin 19 (*Krt19*) promoter but this model failed to develop PDAC [186]. Very recently, a mouse model was developed in which the oncogenic $Kras^{G12D}$ was placed in the endogenous *Kras* locus, and inducible Cre recombinase was expressed under the control of the *Krt19* promoter. The pancreata of these animals failed to develop PDAC but they developed a small number of mucinous metaplasias with characteristics of early stage PanINs [187].

Several recent models have shown that activation of KRas specifically in acinar cells during embryonic stage induces the formation of PanIN and the development of invasive ductal carcinoma, demonstrating that indeed PDAC can arise from acinar to ductal transdifferentiation [89, 91, 188]. However, activation of this oncogene in differentiated acini of adult mice failed to induce any neoplastic lesion [91], or induces the formation of PanINs only in some of the cells that express mutant Kras but these do not progress into the invasive carcinoma stage [89, 90]. These observations suggest that adult acinar cells are refractory to KRas driven neoplastic transformation.

Further experiments by Guerra and colleagues have shown that induction of chronic pancreatitis by caerulein treatment, in adult animals expressing the oncogene under the control of the Elastase acinar-specific promoter, leads to the development of the full spectrum of PanINs and PDAC [91]. These are very important results clearly demonstrating that, as it happens in the human disease, PDAC results from the combination of both genetic factors

and extrinsic factors that produce tissue injury, such as the inflammatory damage observed during pancreatitis.

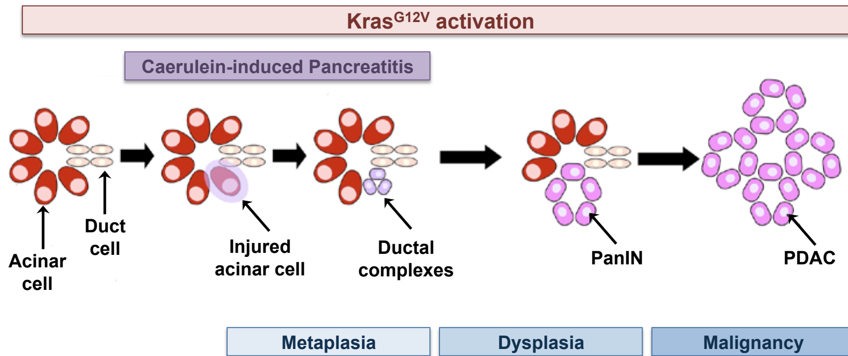


Figure I10. Chronic pancreatitis induces the development of PDAC in adult pancreatic acinar cells expressing *Kras*^{G12V} oncogene [91]. In the mature pancreas, acinar cells appear to tolerate expression of activated *Kras*^{G12V} oncogene without effect. Chronic injury caused by caerulein treatment dramatically sensitizes the adult pancreas to *Kras*^{G12V}-driven dysplasia, possibly by evoking the formation of metaplastic tubular complexes, derived from the transdifferentiation of mature acinar cells. Such complexes would be uniquely sensitive to the effects of the oncogene, resulting in the development of pancreatic intraepithelial neoplasia (PanIN) lesions and invasive pancreatic ductal adenocarcinoma (PDAC)[91]. *Adapted from Murtaugh and Leach, 2007* [189].

Friedlander et al. targeted oncogenic KRas to acinar cells and obtained infrequent PanINs whose progression to PDAC was significantly accelerated by induction of pancreatitis. Surprisingly, in this study it was found that also endocrine cells could be the origin of PDAC: a subpopulation of adult Pdx1-expressing cells was susceptible to oncogenic KRas-induced transformation in the absence of tissue injury, whereas adult insulin-expressing endocrine cells were refractory to the effects of the oncogene. Nevertheless, when challenged with chronic caerulein treatment, adult insulin^{+} cells were able

to give rise to PDAC [188]. This is an unexpected result since previous studies using lineage tracing of insulin$^+$ cells in caerulein induced pancreatitis, showed no evidence of β-cell contribution to metaplastic ductal lesions [88].

III.4.2. The role of tumor suppressors

The Pdx1-Cre;LSL-KRas^{G12D} model has been combined with loss of function alleles of the tumor suppressors most commonly inactivated in human PDAC. Interestingly, in the absence of oncogene activation, mice with a pancreas-specific inactivation of *Ink4a/Arf* or *Trp53* do not develop pancreatic neoplasia. However, when these mutations are combined with oncogenic Kras, the mice develop PDAC with an accelerated progression in comparison with mice harbouring only Kras mutations in the pancreas[190-192]. Different combinations of mutations in tumor suppressor genes, in conjunction with Kras^{G12D} expression, produce tumors with varying spectra of clinical and histological features. While tumors of all genotypes are locally invasive and show micrometastases, gross metastases appear to be restricted to strains with heterozygous tumor suppressor deletions[190-192]. This may reflect the fact that mice with homozygous inactivation develop multifocal tumors which result in a rapidly lethal tumoral burden, whereas heterozygous mice have a longer latency which allows clonal maturation, progression, and metastasis [125]. Regarding tumor histology, *Trp53* loss is associated with a higher prevalence of well-differentiated ductal adenocarcinoma. Conversely, undifferentiated sarcomatoid histology, a feature of the Ink/Arf model, is significantly reduced in p53-deficient models [191, 192]. In humans, ductal adenocarcinoma histology predominates, and the sarcomatoid subtype is an uncommon variant of PDAC with more aggressive clinical behavior. These mouse models collectively recapitulate these histologic variants, although at different frequencies from those seen

in spontaneous human tumors. Overall, these observations suggest that eliminating different tumor suppressor genes can influence the cell differentiation phenotypes of the resulting tumors [125].

Mouse models have also been developed to analyze the involvement of the Tgfβ pathway in PDAC development either by targeting Smad4 [193-195] or Tgfβ receptor 2 (Tgfbr2) [196]. Conditional inactivation of *Smad4* or *Tgfbr2* in the pancreas had no discernable impact on pancreatic development or physiology but when combined with the mutant *KRas*^{G12D} allele, there was induction of PDAC with a shorter latency than the observed in the Pdx1-Cre;Kras^{G12D} model alone. The Tgfbr2 model developed PanINs and well differentiated PDAC with 100% penetrance and with a very fast progression rate[196], whereas Smad4 loss yielded tumors that retained a more differentiated ductal histopathology, either intraductal papillary mucinous neoplasia (IPMNs) [193, 195] or mucinous cystic neoplasms (MCNs) [194]. The more aggressive behavior of the Tgfbr2 model and the distinct tumor phenotypes of the two models could reflect the role of Smad4 in mediating signals from other classes of TGF-β family receptors, such as activin or BMP[193].

An important difference between the mouse models and the human disease is that, in the genetically modified mice, the activation of KRas and loss of tumor suppressor function occur simultaneously and in a large fraction of epithelial cells, while in human PDAC progression it is unlikely that both genetic changes occur at the same time and the proportion of mutant cells in the pancreas is very low (except for patients with inherited mutations in tumor suppressors). These differences imply distinct roles of selection pressure for accumulation of tumor suppressor losses as well as distinct "population effects". The observation that enforcing tumor

suppressor loss, out of the order that may occur in the spontaneous development of human disease, changes the course of PDAC development suggests that the disease depends on specific and sequential tuning of signaling pathways, in a similar fashion to normal development [197].

III.4.3. Developmental signaling pathways in PDAC

Characterization of both human and murine tumors indicates that disease progression depends not only on the loss of tumor suppressors, but also on the development of abnormal activity of other signaling pathways, namely those involved in developmental processes such as Notch, Hedgehog and the Wnt/β-catenin pathways.

Miyamoto et al. first demonstrated that human PDAC and PanINs overexpress members of the Notch pathway, including the downstream target HES1 [7]. De la O et al. confirmed these results in mice, where they found that Notch pathway activation promotes both initiation and dysplastic progression of acinar-derived PanINs with *KRas* mutation, providing an explanation for how a characteristically ductal tumor can arise from acinar cells [89]. More recently, a study using the $Kras^{G12D}$ knockin model in a p53 hemizygous background showed that inhibition of γ-secretase activity reduced the number of PanIN lesions and suppressed tumor formation [198]. Thus, it remains unclear precisely how and when Notch might act in the acinar-duct-PanIN conversion and the pathway may prove to have divergent effects depending on the cellular context [199].

Hedgehog (Hh) signaling has been shown to be a mediator of pancreatic cancer tumorigenesis. Hh ligand is highly expressed in human PDAC, being detectable throughout pancreas progression [200]. Moreover, pancreata of Pdx1-Shh mice, in which Sonic hedgehog (Shh) is misexpressed in the pancreatic endoderm, developed PanIN lesions which contained *Kras*

mutations [200]. Nevertheless, epithelial PDAC cells do not respond to Hh ligand and are refractory to ligand inhibition. In fact, Hh signaling in PDAC seems to act in a paracrine manner, in which the ligand produced by epithelial cancer cells activates the pathway through Patched (Ptch) and Smothened (Smo) in cells of the stroma, which include cancer-associated fibroblasts, infiltrating bone-marrow derived cells and endothelial cells[201, 202]. This pathway emerged as a good candidate for therapy when, recently, it was demonstrated that inhibition of Hh signaling through an inhibitor of Smo increases the tumor cell availability of gemcitabine as a result of a stromal collapse that is accompanied by reduced tumor growth in a KRas-driven PDAC model [203].

β-catenin has been found to accumulate in human PanINs and PDAC, both in the nucleus (10-60%) and cytoplasm (25-65%) [204, 205]. Nevertheless, there is debate about the role of β-catenin signaling in PDAC, since canonical activation of the Wnt pathway is found only in a small subset of pancreas cancer cell lines [206] (Pujal et al, 2006). Several experimental models have been used to study the effect of β-catenin/Wnt signaling in the pancreas. When a stabilized form of β-catenin is expressed in all pancreatic progenitors using an early Pdx1-driven promoter, mice develop a severe exocrine agenesis with formation of large cysts and postnatal lethality. By contrast, stabilized β-catenin expressed through the control of a late Pdx1 promoter restricted β-catenin expression to acini and islets, resulting in a post-natal expansion of the exocrine pancreas [207]. Similarly, pancreas-specific inactivation of the tumor suppressor Adenomatous Polyposis Coli *(APC)* using late Pdx1-Cre mice resulted in an age-dependent accumulation of β-catenin and pancreatomegaly [208]. In both these models increased β-catenin in aging mice failed to lead to tumors [207, 208]. Heiser and colleagues have shown that activation of β-catenin using a Ptf1a-Cre driver resulted

not only in acinar cell proliferation but also in the development of large benign tumors, resembling human pseudopapillary neoplasms. Unexpectedly, mice in which β-catenin and oncogenic Kras were concurrently activated developed distinct tumors with a cribriform morphology, that do not resemble PanIN lesions or PDAC [209]. More recently, Morris et al. have shown that stabilized β-catenin signaling antagonizes the ability of mutant KRas to reprogram acinar cells into PanINs [210] . Altogether, these results show that β-catenin is a crucial mediator of exocrine cell proliferation in a temporal and cell type-specific manner. Moreover, they highlight the fact that developmental signaling pathways must be tuned to appropriate levels at key time points during transformation to specify the PanIN-PDAC lineage progression[197].

III.5. Pancreatic cancer stem cells

Tumors are heterogeneous entities, constituted by different populations of cells. The cancer stem cell model supports the hypothesis that growth, regrowth and metastasis is driven by a specific subpopulation of tumor initiating cells. These cancer stem cells would have self-renewal and multipotent capacity to generate progeny of the various differentiation states that constitute the bulk of the tumor [211]. Pioneering work in this area originated from studies of leukemia stem cells [212] and later, the existence of cancer stem cells was demonstrated in solid tumors, specifically in human breast [213] and brain tumors[214].

Pancreatic cancer stem cells were first identified by Li et al. being defined by the expression of the cell surface markers CD44, CD24 and ESA. These cells represented only 0.2-0.8% of all pancreatic cancer cells, were highly tumorigenic, and possessed the ability to both self-renew and produce

differentiated progeny that reflected the heterogeneity of the patient's primary tumor [215].

A second study by Hermann and colleagues found that in primary pancreatic cancers and pancreatic cancer cell lines, CD133 expression also identifies cells with enhanced proliferative capacity and tumorigenic potential that are highly resistant to chemotherapy. In the invasive front of pancreatic tumors, a distinct subpopulation of $CD133^{+}CXCR4^{+}$ cancer stem cells was identified that determines the metastatic phenotype of the tumor[216]. More recently the same authors have also shown that the combined blockade of Hh and mTOR signaling, together with standard chemotherapy, was capable of eliminating the $CD133^{+}CXCR4^{+}$ cells both *in vitro* and in mouse models of pancreatic cancer[217].

Interestingly, only an approximately 14% overlap was found between $CD44^{+}CD24^{+}ESA^{+}$ and $CD133^{+}$ cells [216]. These findings suggest that more than one set of specific cell surface markers may enrich for pancreatic cancer stem cell populations and that a better expression profile to identify pancreatic cancer stem cells needs to be developed. In addition, it is mandatory that marker studies are complemented with functional assays to properly determine the stem cell behavior.

III.6. Uncommon pancreatic tumors

Recently, there has been an increased recognition of pancreatic neoplasms other than PDAC. These rare tumors have been less extensively studied but they exhibit an overall better prognosis, and display specific genetic features, thus meriting further analysis. Regardless of the uncertainties regarding the cellular origin of distinct tumors, current classifications are based on the morphological features of each type of neoplasm [218].

III.6.1. Intraductal papillary mucinous neoplasms

Intraductal papillary mucinous neoplasms (IPMNs) are exocrine tumors that arise inside the ductal system. These neoplasms are formed by tall and columnar epithelium, usually with papillary projections and containing mucin-producing cells [219]. IPMNs may have a variable degree of neoplastic features ranging from adenoma, to dysplasia, to carcinoma in situ or to invasive carcinoma [220]. Once barely recognized and thought to be very rare, today they represent the most common cystic neoplasms of the pancreas and have attracted recent interest because their incidence appears to be on the rise[218]. There are 2 subtypes of this disease, the branch duct IPMNs and the main-duct IPMNs, which have specific clinical, morphological and epidemiologic features. Most branch-duct IPMNs were found to be asymptomatic and invasive cancer was present only in 11% of the cases while main-duct IPMN patients were symptomatic with invasive cancer being present in 48% of cases [221]. A comparison of recent reports showed a 5-year survival rate from 77-100% for patients having non-invasive IPMNs compared to 43-65% in those patients with invasive disease [219]. IPMNs are a ductal type neoplasia and thus express ductal lineage markers. They also share the genetic alterations that characterize PDAC such as mutations in *KRAS* and *TP53* and loss of *p16/INK4A*, but interestingly most of the cases retain *SMAD4* [220]. The similarities of IPMNs with PanINs and PDAC has made that some authors would consider these neoplasms as PDAC precursor lesions and not an independent type of tumor [125].

III.6.2. Mucinous cystic neoplasm

Mucinous cystic neoplasms (MCNs) have an epithelial lining made of columnar mucin producing cells [219]. These tumors are distinguished from IPMNs because they do not communicate with the ductal system, contain

an ovarian-like stroma, and affect almost exclusively women[220]. These tumors have a good prognosis as only 11% of cases develop invasive carcinoma and 5-year survival rate of non-invasive MCNs approaches 100%[221]. As IPMNs, these neoplasms also present mutations in *KRAS* and *TP53* and have been considered by some authors as PDAC precursor lesions[125].

III.6.3. Acinar Cell Carcinoma

Acinar cell carcinomas (ACCs) are solid, differentiated tumors with little stroma, which produce digestive enzymes such as trypsin, lipase or chymotrypsin, and have higher mitotic rates than normal acini and display abnormal gland architecture. These carcinomas may arise in any part of the organ, tend to be unifocal and well circumscribed, and are usually large in size [222]. ACCs account for approximately 1-2% of adult pancreatic neoplasms and 15% of pediatric tumors and are highly aggressive tumors with a mean survival of 18-19 months [223].The genetic alterations present in these tumors are distinct from the ones found in PDAC [224] and 24% present alterations in the Wnt / β-catenin signaling pathway, either through activating mutations in β-catenin or truncating mutations of the *APC* gene[225].

III.6.4. Pancreatoblastoma

Pancreatoblastoma is a malignant epithelial neoplasm with a prominent acinar differentiation. It differs from ACC particularly because of the presence of squamoid nests, extensive lobulation, a stroma with high cellular density, and the presence of some endocrine and ductal components. As ACC, pancreatoblastomas may arise in any location of the pancreas, are usually well circumscribed, and are large in size. It is the most common pancreatic neoplasm in childhood, being present in approximately

25% of pediatric pancreas cancer cases[223]. A genetic signature similar to ACCs is found in these tumors, with mutations in the β-catenin in 50-80% of the cases [226].

III.6.5. Solid Pseudopapillary Neoplasm

Solid pseudopapillary neoplasms (SPNs) are characterized by having a solid pattern of growth with a cystic component and pseudopapillae. These tumors do not show any type of pancreatic cellular differentiation and their cellular origin is currently unknown. SPNs are uncommon tumors, accounting for 2-5% of all pancreatic malignancies and present a higher incidence among young women [223]. The most common features of these tumors are the loss of E-cadherin and the translocation of β-catenin to the nucleus [227].

III.6.6. Pancreatic Endocrine Neoplasm

Pancreatic Endocrine Neoplasms (PENs) present a neuroendocrine phenotype. Functional pancreatic endocrine tumors are constituted by well differentiated hormone-secreting cells, being named after the hormone they produce. For instance, insulinomas produce insulin, gastrinomas produce gastrin, glucagonomas produce glucagon, VIPomas produce vasoactive intestinal peptide, and somatostatinomas produce somatostatin. In these neoplasms, patients present with symptoms caused by hormone overproduction. Additionally, there is a subset of non-functional tumors which do not lead to clinical symptoms, but tumor cells express neuroendocrine markers such as chromogranin-A and somatostatin receptors [219].

These tumors account for 1-2% of all pancreatic tumors and insulinomas are the most common, accounting for 42% of PEN cases. Approximately 50-

80% of PENs recur and metastasize, and the prognosis of functional PENs tends to be better than that of non-functional PENs [223]. Studies have shown that alterations present in PENs are very distinct from those in exocrine tumors and mutations in *KRAS*, *TP53*, *p16/INK4A* and *SMAD4/DPC4* are virtually absent [218]. A recent study has shown that the genes *DAXX/ATRX*, *MEN1*, and genes belonging to the mTOR pathway are frequently altered in PENs [228].

IV. The tumor suppressor p53

IV.1. Introduction

TP53, encoding for the p53 protein, is the most frequently altered gene in human cancer, being mutated in approximately 50% of tumors. In many other tumors the p53 pathway is inactivated indirectly through binding to viral proteins or as a result of alterations in other genes whose products regulate p53 [229].

p53 was discovered in 1979 as a cellular partner of the simian virus 40 T-antigen that elicits an immune response in mice [230-232]. First attempts for cloning the DNA used tumoral cells rather than normal tissue, which led to the initial erroneous classification of p53 as an oncogene [233-235]. It was not until 1988, when the cDNA of wild type p53 was cloned from normal murine cells, that it became clear that p53 is in fact a tumor suppressor gene [236, 237]. Only then it was realized that *TP53* was frequently mutated in tumors and that the mutated form could exert cancer-promoting effects by dominant negative inactivation of wild type p53 as well as oncogenic gain of function activities [238].

Subsequent studies clearly established p53 as a *bona fide* tumor suppressor. It was first described to be lost in human cancer by Vogelstein and colleagues who showed that *TP53* was frequently inactivated by mutation, deletion or a combination of both in human colorectal tumors [239]. An extensive number of following studies revealed that indeed p53 was lost in around half of all human tumors [238]. Furthermore, it was found that germline mutations in *TP53* were responsible for the hereditary Li-Fraumeni Syndrome, causing the early onset of multiple types of cancer [240, 241] and *Trp53* knockout mice were found to develop cancer with a high

penetrance[242]. Altogether these findings have placed p53 as the most extensively studied tumor suppressor [238], which still today is the matter of many new publications and whose new functions are still being unraveled.

IV.2. The p53 gene family

p53 is a transcription factor that binds the DNA through a p53 responsive element, transactivating genes that mediate essential p53-dependent functions in the cells, such as cell cycle arrest, DNA repair or apoptosis.

In 1997 and 1998, two genes possessing a remarkable similarity with *TP53* were identified, being named *TP63* [243] and *TP73* [244, 245]. The hallmark features of the p53 protein - an N-terminal transactivation domain (TAD), a core domain for DNA-binding (DBD) and a C-terminal oligomerization domain (OD) - are shared by both p63 and p73, by which these three proteins constitute the p53 family of transcription factors [246]. The high level of sequence conservation between p63, p73 and p53 proteins, particularly in the DBD, allows p63 and p73 to transactivate p53-responsive genes, being able to induce cell cycle arrest or apoptosis. However, they are not functionally entirely redundant and each p53 family member has its own unique functions [247].

IV.2.1. p53 protein structure

The human *TP53* gene is located at chromosome 17p13.1. It is composed of 19,198 nucleotides and 11 exons. The coding sequence begins in the second exon and ends in the last exon, encoding 393 amino acids that constitute the full-length p53 protein [248]. p53 protein is active as a tetramer formed by four identical peptides. The N-terminal region consists of an intrinsically disordered transactivation domain (TAD) and a proline-rich region (PRR). It is followed by the central, folded, DNA-binding core domain (DBD) that is

responsible for sequence-specific DNA binding. This domain is connected, via a flexible linker, to a short tetramerization domain that regulates the oligomerization state of p53 (OD). At its C-terminus (CTD), p53 contains the so-called regulatory domain, an unfolded region rich in basic amino acids which binds DNA nonspecifically [249, 250].

The N-terminal region of p53 is natively unfolded and consists of an acidic TAD, which is often further subdivided into two subdomains TAD1 (residues 1–40) and TAD2 (residues 40–61) and a proline-rich region (residues 64–92). The TAD is a promiscuous binding site for a multitude of interacting proteins, such as components of the transcription machinery, the transcriptional co-activators p300/CBP (CREB-binding protein), and the negative regulators MDM2 and MDMX [249, 250].

p53 DBD consists of an immunoglobulin-like β-sandwich that provides the basic scaffold for the DNA-binding surface and can be subdivided into two structural motifs that bind to the minor groove and major groove of target DNA [249, 250]. p53 recognizes and bind to its DNA recognition elements (REs) that are located, in most cases, within a few thousand base pairs of the transcriptional start site. The p53 consensus sequence is composed of two pairs of head-to-head arranged pentamers 5′-RRRCWWGYYY-3′, where R is a purine, Y a pyrimidine, W is either A or T (adenine or thymine), G is guanine and C is cytosine. The p53-binding site in the genomes of many organisms is composed of a half-site RRRCWWGYYY followed by a spacer, usually composed of 0–21 base pairs, which is then followed by a second half-site RRRCWWGYYY sequence. The spacer sequence as well as the pentamers' mutual orientation are important determinants that may influence p53 binding ability and activity [251, 252].

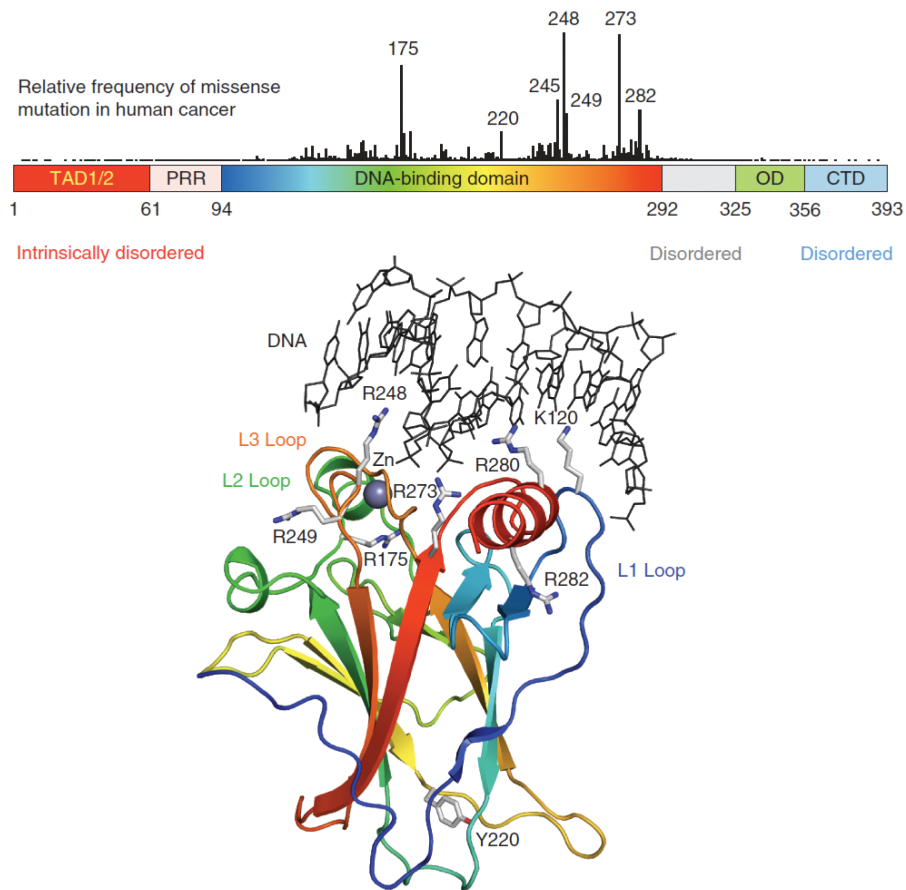


Figure I11. p53 protein structure. p53 contains a natively unfolded N-terminal transactivation domain (TAD), which can be subdivided into the subdomains TAD1 and TAD2, followed by a proline-rich region (PRR). The structured DNA-binding domain (DBD) and oligomerization domain (OD) are connected through a flexible linker region. The regulatory domain at the extreme C terminus (CTD) is also intrinsically disordered. The vertical bars indicate the relative missense-mutation frequency in human cancer for each residue, showing that most cancer mutations are located in the DBD. The structure of the DNA-binding domain is shown as a ribbon representation and colored with a rainbow gradient from the amino-terminus (blue) to the carboxyl terminus (red). Sites of cancer hotspot mutations and essential DNA contacts are shown. *Adapted from Joerger and Fersht, 2010* [250].

Among more than 18000 currently known mutations in p53 that result in either partial or complete loss of its wild type functions, amino acids within the p53 DBD are by far the ones that are most frequently mutated in human cancer [252]. The mutations situated in the DBD can disrupt p53-specific DNA binding by several possible ways (discussed bellow).

The extreme C-terminus of p53 is intrinsically disordered but may undergo local disorder-to-order transitions upon binding to other proteins or to nonspecific sequences in DNA. The C-terminal region of p53 is subject to extensive post-translational modifications, including acetylation, ubiquitination, phosphorylation, sumoylation, methylation, and neddylation, that regulate p53 function and cellular protein levels [249].

IV.2.2. The p53 homologs: p63 and p73

p63 and p73 are two structural and functional homologs of p53 that, as a result of sharing similar domain architecture with it, can form oligomers, bind DNA and transactivate p53-responsive genes [253].

The three major protein domains are highly conserved between the three family members. The TAD is the least conserved, with 22% identity between p63 and p53 and 30% between p73 and p53. The DBD of p63 and p73 are 60% and 63% identical with p53 respectively. In addition, the residues of p53 that directly interact with DNA are identical in p63 and p73 [253]. Consequently, both p63 and p73 can bind to canonical p53 DNA-binding sites and activate transcription from p53-responsive promoters, thus inducing cell cycle arrest, apoptosis and cellular senescence [243, 244, 254-256]. The C-terminal OD of p53 is 38% identical with p63 and p73[253].

Additionally, the p63 and p73 genes have two distinct promoters. The first promoter yields a full-length protein, the isoforms TAp63 and TAp73,

containing a TAD, and that generally behave similarly to p53 in terms of overlapping target promoters and biological functions. The second promoter located within intron 3, gives rise to N-terminally truncated proteins (DN isoforms). The latter lack the N-terminal TAD and, as a result, these isoforms are transcriptionally inactive, acting as dominant negative inhibitors of the active family members [247]. As a consequence, full-length and truncated isoforms of the p53 family generally exhibit reciprocal biological functions: while the full-sized isoforms TAp63 and TAp73 are functionally similar to p53, promoting cell cycle arrest and/or apoptosis in response to stress, the truncated DNp63 and DNp73 isoforms are thought to have pro-survival, anti-p53 functions [257].

Alternative splicing at the C terminus generates three splice variants (α,β,γ) within each group (TA and DN) of p63 and p73 isoforms. The γ-isoform is most similar to p53, with a slight extension containing a small polyglutamate stretch found in all p63/p73 proteins. The β- and α-isoforms are longer, with the latter containing a steric α-motif (SAM)-like sequence, thought to mediate protein–protein interactions [246]. In addition to the overlapping functions shared with p53, the existence of extensive structural variability within the family determines unique roles for p63 and p73. In fact, in the absence of stress, the most important role of these p53 family members is the regulation of differentiation and development [257].

IV.3. Regulation of the p53 pathway

p53 pathway activation has two major outputs: cell cycle arrest and/or apoptosis, playing an important role in stress responses. Thus, p53 is generally “off” in normal cells. Multiple mechanisms exist to negatively control p53, including the regulation of protein activity, stability and subcellular localization. p53 regulatory proteins include ubiquitin ligases

that control protein stability, kinases and acetylases involved in post-translational modifications of p53, transcriptional co-activators that modulate p53 transcriptional activity and many others[168].

IV.3.1. Control of p53 levels: the p53-MDM2 feedback loop

The main mechanism by which p53 levels are kept low in normal cells involves MDM2 and other proteins of its family, which bind to the N-terminal domain of p53 and sterically block p53 transactivation. MDM2 is an E3 ubiquitin ligase that also regulates the activity of p53 by ubiquitinating it, transporting it to the cytoplasm, and promoting its degradation by the proteasome. p53 and MDM2 form a negative feedback-loop in which MDM2 is a direct transcriptional target of p53, thus promoting p53 degradation and quenching p53 cellular activity. Exposure of cells to stress reduces sumoylation of MDM2, and causes an increase in self-ubiquitination and degradation, favoring p53 stabilization. There is also evidence that post-translational modification of MDM2 destabilizes its interaction with p53 to contribute to p53 stabilization in response to stress[238, 258].

MDMX/MDM4 is another player in this regulatory loop. Like MDM2, MDMX binds to the transactivation domain of p53 and inhibits its activity. Although MDMX lacks E3 ubiquitin ligase activity, it contributes to p53 degradation through dimerization with MDM2, augmenting its ubiquitination activity [238].

The control of p53 stabilization and activation in response to stress stimuli is very complex, involving direct phosphorylation of several molecules including p53 itself, MDM2 and MDMX. Distinct types of stresses lead to the activation of protein kinases including ATM, ATR, JNK, c-ABL, PI3K and

AKT, all of which influence the stability and/or activity of p53. The whole process of p53 activation is sophisticated and fine-tuned to ensure that this molecule is maintained at low levels in normal conditions while being activated upon appropriate stimuli [258].

IV.3.2. Stress induced activation of p53

Activation of p53 is driven by a wide variety of stress signals that a cell may encounter during malignant progression and which include DNA damage, oncogene activation, telomere erosion, nutrient deprivation and hypoxia. These signals do not engage p53 through the same pathways, instead they use distinct mechanisms to induce its stabilization and activation [168].

DNA damage was the first type of stress found to activate p53 by which this protein has been widely regarded as the "guardian of the genome" [259]. p53 is very sensitive to DNA damage, activating a response that is thought to contribute to tumor suppression by either allowing for repair or by eliminating cells with potentially oncogenic alterations [168].

DNA damage efficiently induces the p53 response through the activation of a cascade of Ser/Thr kinases that involve ATM, ATR, CHK1 and CHK2, which phosphorylate p53. The original concept of rather strict dependency of CHK1 on ATR, and CHK2 on ATM, has recently been challenged by reports of various crosstalks among these kinases [260]. The activation of the ATM/ATR/CHK1/CHK2 cascade results in the phosphorylation of both p53, MDM2, and MDMX, inhibiting the interaction between p53 and MDM2 and enhancing the degradation of both MDM2 and MDMX. In addition to the stabilization of p53, DNA damage-induced kinases may also play a role in activating p53 as a transcription factor, in part by promoting N-terminal phosphorylation and C-terminal acetylation of p53 [261]. This signaling

pathway is permanently activated in human cancer, suggesting that the cancerous state is intrinsically associated with DNA damage [262].

Characterization of mice that have a mutant form of p53, which cannot be phosphorylated by ATM/ATR/CHK1/CHK2, revealed that these animals have a milder phenotype than p53-null mice and maintain some DNA damage induced p53-dependent functions [263]. Therefore, this pathway is not the only responsible for the DNA damage response (DDR), and other pathways are also thought to play a role in activating p53 in this context, such as the p38, JNK/SAPK and c-ABL pathways [262] .

Telomere shortening is another important stimulus that activates p53. Dysfunctional telomeres are recognized by the cell as DNA double strand-breaks leading to p53 activation through the DDR pathway [264].

Oncogenic signaling has also been shown to activate p53 and, similar to DNA damage, it is universally present in cancer.

The key mediator of oncogene induced p53 activation is p14/ARF, which interacts with MDM2 and sequesters it, inhibiting its p53-ubiquitin ligase activity. This results in stabilization of p53 and consequent increase in this protein's activity [262]. Many transcription factors activate ARF in response to oncogenic stress, most notably DMP1, which has been seen to act downstream of RAS signaling [265]. Mice lacking Arf have a tumor-prone phenotype, suggesting that the inactivation of the ARF-p53-MDM2 pathway has a critical role for cancer progression. Nevertheless, these animals present a normal DNA damage response, indicating that these are two independent pathways [266, 267]. More recent studies using mouse models have shown that p53 is unable to protect from cancer in the absence of Arf, even when overexpressed, and despite the existence of an efficient

response to DNA damage, revealing the essential function of ARF-mediated activation of p53 in tumor suppression [268, 269].

Several physiological stimuli have been shown to activate p53 through the **ribosomal stress** pathway, such as serum starvation and cell-cell contact inhibition. A number of ribosomal proteins such as L4, L11 and L23 have been shown to bind and inhibit MDM2 [270-273], and to enhance MDM2-mediated degradation of MDMX [274] thereby stabilizing and activating p53. DNA damage can also lead to the degradation of the ribosomal protein L37, activating p53 through the L11/MDM2 pathway [275].

Hypoxia also activates p53, stimulating p53-dependent apoptosis. Hypoxic or anoxic conditions promote the stabilization of HIF1α (Hypoxia inducible factor), by disrupting its interaction with VHL (Von Hippel-Lindau). HIF1α is able to bind p53, promoting its stabilization. VHL can also activate p53, by promoting its phosphorylation and acetylation [261].

IV.4. The tumor suppressor functions of p53

The p53 protein has been extensively studied and its tumor suppressive role, which involves p53 ability to inhibit cell proliferation in cells subjected to acute stress, is very well established. In this context, p53 has been shown to regulate genes that mediate cell-cycle arrest, senescence and apoptosis. However, recent studies have revealed that apart from these very well understood tumor suppressor functions, p53 can contribute to numerous other aspects of disease and normal life. These include roles of p53 in regulating DNA repair, longevity and ageing, glycolysis, autophagy, the repair of genotoxic stress, invasion and motility, angiogenesis and numerous aspects of differentiation and development [168, 276].

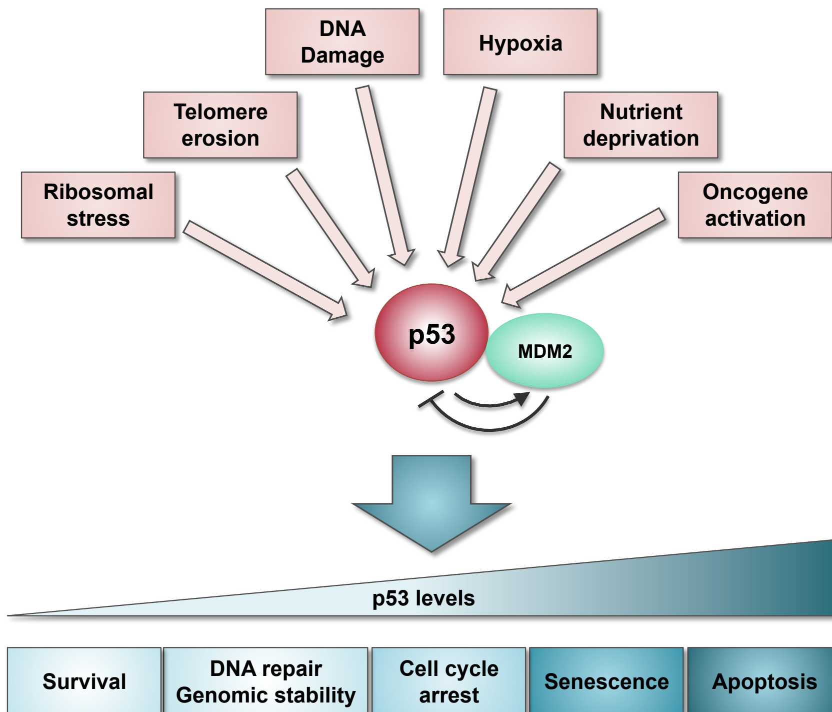


Figure I12. Activation and functions of p53. p53 has an important role in integrating the cellular responses to different types of stresses. Different stress signals are able to induce p53 (pink boxes), resulting in the activation of a number of cellular responses (blue boxes). The p53–MDM2 feedback loop is the 'heart' of the p53 pathway. Under normal conditions, it maintains constantly low steady-state p53 levels and activity. Stress signals would impose on this central loop to release p53 from MDM2-mediated inhibition, increasing p53 protein levels and activity. Activated p53 can then play a role in determining which response is induced through differential activation of target-gene expression. It is thought that p53 responds to conditions of low or constitutive stress, by providing repair functions to mend low levels of DNA damage and thus, contributing to the survival and health of the cell, as well as to the prevention of the acquisition of tumorigenic mutations. By contrast, acute stress that results in a more robust induction of p53, leads to the activation of irreversible tumor suppressive mechanisms, such as senescence and apoptotic cell death thereby leading to the elimination of the damaged cell. *Adapted from Vousden and Lane, 2007* [168].

IV.4.1. Cell-cycle checkpoints

The process of cell division is highly ordered and regulated. Cell-cycle checkpoints exist to ensure appropriate progression before transition into the next cell cycle phase. These are essential control features that ensure the fidelity of cell division and prevent the accumulation of mutations in the DNA and appropriate repartition of genetic material in daughter cells [277].

The role of p53 in cellular growth arrest has been extensively studied, and many of the key upstream regulators and downstream effectors of p53 have been described. It has been shown that mouse embryonic fibroblasts (MEFs) exposed to DNA damage activate p53 which induces a G1 arrest primarily through the transactivation of p21/Waf1/Cip1, a cyclin-dependent kinase inhibitor [278]. Mice deficient for p21 have been shown to undergo normal development but have a defective G1 checkpoint control [279].

Progression of cells from G2 to mitosis is driven by the maturation-promoting factor (MPF), which comprises a complex of cyclin B1 and CDC2. p53 has been shown to induce G2/M arrest by primarily perturbing the function of the cyclin B1/CDC2 complex. Specifically, p53 represses CDC25C, a phosphatase that promotes mitosis, after DNA damage [280]. Additionally, p53 has been shown to transcriptionally activate 14-3-3s after DNA damage. 14-3-3s is a protein that prevents proper nuclear localization of cyclin B1/CDC2 after DNA damage [281].

The activation of the cellular checkpoints by p53 is engaged when the cell is subjected to a low level of stress, allowing the repair and reversal of the damage. This allows cell survival, preventing the acquisition of mutations, and might contribute to overall longevity and normal development. Indeed, p53 can directly activate genes that contribute to DNA repair and to the decrease of oxidative stress. The other two main outcomes of p53

activation are senescence and cell death, which are terminal for the cell. These outcomes are activated when the cell is subjected to an acute stress, allowing for the elimination of a highly damaged cell [168, 276].

IV.4.2. Senescence

Cellular senescence is a permanent form of cell-cycle arrest that was originally described more than four decades ago, when Hayflick and colleagues showed that normal cells had a limited ability to proliferate in culture [282]. Two important hypotheses eventually emerged to describe the biological importance of senescence *in vivo*. In the first, cellular senescence would have a beneficial effect, as it was proposed to be a tumor suppressive mechanism. The second hypothesis suggested that senescence recapitulated ageing or loss of regenerative capacity of cells *in vivo* and, in this context, cellular senescence was considered deleterious. For many years these hypotheses were pursued independently, but as the understanding of the senescence response developed, they coalesced, bringing new insights to the fields of cancer and ageing. However, cellular senescence is not equivalent to the concept of aging. [283]

The notion of oncogene-induced senescence emerged when Serrano et al. showed that normal cells forced to express an oncogenic form of H-Ras, rather than increasing their proliferation, activated a growth arrest that was indistinguishable from senescence [182]. More recently, the occurrence of cellular senescence has been reported to occur *in vivo*, in mouse and human tumors, being associated with pre-malignant stages of neoplastic transformation [179-181, 183]. All these evidences clearly established senescence as an anti-cancer mechanism that provides a barrier to tumor progression.

A hallmark of cellular senescence is an inability to progress through the cell cycle. Senescent cells arrest growth, usually with a DNA content that is

typical of G1 phase, failing to initiate DNA replication despite adequate growth conditions. In contrast to quiescence, the senescence growth arrest is essentially permanent, but nevertheless the cells remain metabolically active [283]. The senescence response involves significant changes in cellular phenotypes. Cells have a large and flattened morphology and show marked but consistent changes in their gene expression profile. Several markers are typically used to identify senescent cells, albeit no markers currently in use are exclusive to the senescent state. These include senescence β-galactosidase (SA-βgal) activity pH 6.0, as well as increased expression of p53 and cell cycle regulators, such as p21 and p27. The products of the *CDKN2A* locus (p16/INK4A and p14/ARF) are also useful markers of senescence *in vivo*. New markers of senescence, found by DNA microarray analysis, such as the basic helix–loop–helix transcription factor BHLHE40/DEC1 and decoy receptor 2 (DCR2) have also been used successfully in the identification of *in vivo* senescence [167].

Senescence can be induced by various stressors including DNA damage (caused both by dysfunctional telomeres or non-telomeric DNA damage), perturbations in chromatin organization, and excessive mitogenic signaling produced by oncogenic stress. Although many different oncogenes and growth regulatory molecules can trigger senescence, the signaling pathways involved are best defined for oncogenic RAS and its effectors. Chronic signaling through the RAS-RAF-MEK-ERK pathway drives senescence, in part, through activation of the p38MAPK-p16/INK4a stress response pathway but also through activation of DNA damage signals [284].

The senescence program is established and maintained by the both the p53 and p16-RB tumor suppressor pathways. These pathways can respond to similar or different stimuli and can show cell-type specificity with respect to

their ability to induce senescence. Stimuli that generate a DDR induce senescence primarily through the p53 pathway whereas some signals, such as oncogenic RAS, engage both pathways. Furthermore, there are species-specific differences: for example, experimental disruption of telomeres primarily engages the p53 pathway in mouse cells but both the p53 and p16–pRB pathways in human cells [283]. Although shown to independently inhibit cell-cycle progression, there is notable cross talk and redundancy between the p53 and p16-RB pathways, underscoring the importance of senescence as a tumor suppressive biological output. p14/ARF can be activated by various senescence signals and, by binding MDM2, it can activate the p53 pathway. p53 can subsequently transactivate p21/WAF1/CIP1, which, in this context, contributes to p53-dependent senescence rather than a reversible checkpoint arrest. p21 can also inhibit cyclin-dependent kinases upstream of the RB tumor suppressor. RB can in turn inhibit E2F, a potent regulator of genes required for cell proliferation. In the RB pathway, deregulation of E2F can also activate p14/ARF and subsequently lead to p53 activation [281, 283].

It is still unclear what dictates whether cells undergo transient (checkpoint arrest) or irreversible cell-cycle arrest (senescence). It may depend on whether or not the senescence signal is sustained, or possibly on the cooperation of secondary events that "lock" the arrest into an irreversible state. In this regard, it was found that RB directs the formation of senescence-associated heterochromatin foci (SAFs) that contain, and are thought to stably silence, E2F target genes [285].

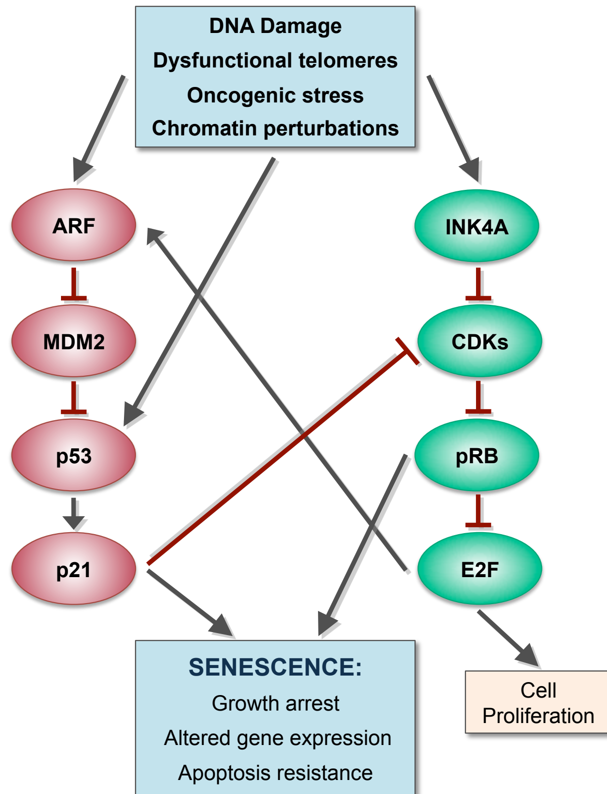


Figure I13. p53 and INK4A-pRB pathways control cellular senescence. Senescence-inducing signals, such as DNA damage, dysfunctional telomeres, oncogenic stress and perturbations in chromatin structure engage either the p53 and/or the INK4A-pRB tumor suppressor pathways. p53 is negatively regulated by MDM2 which facilitates its degradation, and MDM2 is negatively regulated by ARF. Active p53 establishes the senescence growth arrest by inducing the expression of p21, a CDK inhibitor that suppresses the phosphorylation and, hence, the inactivation of pRB. Senescence signals that engage the INK4A–pRB pathway generally induce the expression of INK4A, that also prevents pRB phosphorylation and inactivation. pRB halts cell proliferation by suppressing the activity of E2F, which stimulates the expression of genes required for cell-cycle progression. E2F can also restrain proliferation by inducing ARF, which engages the p53 pathway. The senescence response causes changes in cellular phenotype, which include a permanent arrest of cell proliferation, an altered pattern of gene expression and the development of resistance to apoptosis. *Adapted from Campisi and di Fagagna, 2007* [283].

Recent studies show that there is an increase in various secreted factors in senescent cells, thus providing the basis for a senescence-associated secretory phenotype. These changes involve the up-regulation of extracellular matrix (ECM) degrading enzymes, down-regulation of ECM production, and an overall up-regulation of immune modulating cytokines and chemokines and p53 has been shown to play a significant role in modulating this process [281]. A study be Xue et al. has shown that reactivation of p53 in liver tumors deficient for this protein activated senescence and triggered an innate immune response that targeted the tumor cells, producing a tumor regression [286]. These results evidence that p53-driven senescence can act concomitantly with the immune system to limit tumoral growth.

In addition to influencing the microenvironment, inflammatory cytokines secreted by senescent cells, can have an indispensable role in the establishment and maintenance of the senescence arrest. For example, signaling through the IL-6 and IL-8 (CXCR2) receptors is essential, in a cell-autonomous fashion, for cells to enter senescence in response to oncogenic BRAF or replicative exhaustion, respectively [287, 288]. Similarly, IGFBP7 was also found to be expressed and secreted in response to oncogenic BRAF, acting through autocrine/paracrine pathways to inhibit BRAF-MEK-ERK signaling and induce senescence and apoptosis [289].

IV.4.3. Apoptosis

Apoptosis is a genetically controlled event, defined by molecular and morphological changes that result in the packaging and removal of the dying cell. There are two general signaling pathways that trigger apoptosis and the differences between these pathways dictate how the death signal is transduced [290].

The intrinsic pathway, activated by stresses such as DNA damage or hypoxia, is engaged by the BCL-2 family of proteins, which directly impact on mitochondrial outer-membrane permeabilization (MOMP). After MOMP, cytochrome c is released from the mitochondrial intermembrane space, causing APAF1 oligomerization and resulting in formation of the apoptosome. This complex, in turn, recruits and activates procaspase-9, which then activates executioner caspases-3 and 7. These caspases are responsible for the apoptotic hallmarks, such as chromatin condensation, plasma membrane asymmetry, and cellular blebbing [290]. A family of proteins with structurally conserved domains, known as the BCL2-homology (BH) domain, has a central role in the intrinsic apoptotic pathway. Two of these BH-domain proteins, BAX and BAK, function to promote apoptosis by regulating mitochondrial membrane potential. Anti-apoptotic BH2 proteins, such as BCL-2 and BCL-XL, negatively regulate BAX and BAK, whereas a further group of these proteins, the BCL2-homology domain-3 (BH3)-only proteins, such as PUMA (p53-up-regulated modulator of apoptosis) and NOXA, control these survival proteins[168].

The extrinsic pathway is driven by death-receptor cross-linking by death ligands. For example, tumor necrosis factor (TNF) binds to its death receptor, TNFR1, which causes the recruitment of adaptor molecules, TRADD (TNFR1-associated death domain) and FADD (FAS-associated death domain), and the binding and activation of procaspase-8 molecules. Once caspase-8 is active, it can activate executioner caspases[290].

Although p53 has been implicated in both pathways, it predominantly seems to influence the intrinsic pathway [168]. p53-mediated apoptosis involves the coordination of both transcription-dependent and transcription-independent functions of p53. In the nucleus, p53 can

transactivate numerous genes involved in apoptosis, including the pro-apoptotic genes BAX, PIG3, Killer/DR5, FAS, p53AIP1, PERP, and the BH3-only proteins NOXA and PUMA [251].

p53 also has cytosolic activities that can induce apoptosis in a transcription-independent manner. Specifically, in response to various cell death signals, p53 rapidly localizes to the mitochondria where it can induce MOMP, thus leading to the release of pro-apoptotic factors. p53 can interact with the anti-apoptotic proteins BCL-2, BCL-XL and with the pro-apoptotic protein BAK at the mitochondria, and has been suggested to act like a BH3-only protein, either as a direct activator of BAX and/or BAK, or as a derepressor [291].

More recently, the transcription-dependent and transcription-independent mechanisms of p53 have been linked through the p53 apoptotic target gene PUMA. In response to cellular stress, p53 transactivates PUMA, which then translocates to the mitochondria, where it can bind BCL-XL protein, releasing p53 to activate BAX [292]. These data suggest that the transcription-dependent component of the p53 network is essential for the induction of apoptosis and PUMA plays a critical role in this process. In fact, PUMA seems to be an essential p53 effector in the apoptotic pathway activation but, surprisingly, PUMA-null mice are not overtly tumor-prone. This illustrates the complex nature of the p53 network, suggesting that simultaneous inactivation of multiple p53 effector functions is critical for its role in tumorigenesis [281].

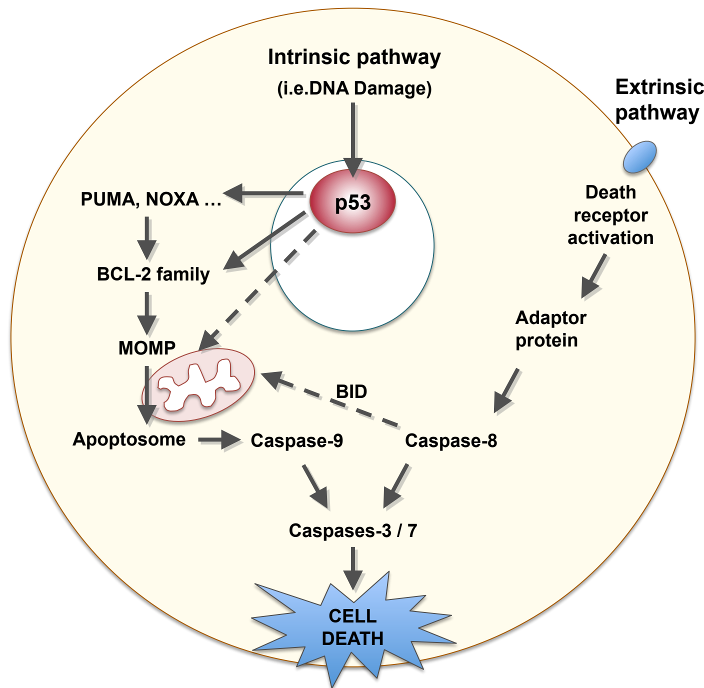


Figure I14. Pathways regulating apoptosis. Apoptotic signals can engage two main pathways: the **extrinsic pathway**, which is induced through the activation of cell-surface receptors; and the **intrinsic pathway**, which responds to stress signals. The **intrinsic pathway** is engaged by cellular stresses, such as DNA damage, and directly impacts on the BCL-2 family of pro- and anti-apoptotic proteins. p53 predominantly influences the intrinsic pathway, by direct transcriptional activation of the BH3-only pro-apoptotic proteins PUMA and NOXA, and of the pro-apoptotic BCL-2 family of proteins, such as BAX and BAK. In the cytoplasm, p53 associates with the anti-apoptotic BCL-2 or BCL-XL proteins, which can release sequestered BH3-only proteins. Once the appropriate repertoire of BCL-2 proteins has been engaged, they elicit mitochondrial outer membrane permeabilization (MOMP), formation of the apoptosome and consequent pro-caspase-9 activation. Executioner caspases-3 and -7 are then activated by caspase-9-dependent cleavage. The **extrinsic pathway** requires the ligation of death receptors by death ligands, which results in the assembly of adaptor molecules and pro-caspase-8 activation. Again, executioner caspases-3 and -7 are then activated by caspase-8. BID (BH3-interacting-domain death agonist) can also be activated by caspase-8 leading to co-engagement of the intrinsic pathway. *Adapted from Chipuk et al., 2006* [290].

IV.5. p53 mutations and cancer

Somatic *TP53* mutations occur in almost every type of cancer at rates from 38-50% in ovarian, esophageal, colorectal, head and neck, larynx, and lung cancers to about 5% in primary leukemia, sarcoma, testicular cancer, malignant melanoma, and cervical cancer. Nevertheless, in cancers with low mutation rates, p53 is often inactivated by alternative mechanisms[293].

The majority (86%) of p53 mutations cluster between codons 125 and 300, corresponding to the DNA binding domain. Here, there are several hotspots, such as the codons 175, 248, and 273, which account for most of the somatic mutations found in human cancers [293]. Strikingly, the vast majority of cancer associated *TP53* mutations actually lead to production of the full length protein, typically with single amino acid substitutions which render the protein resistant to degradation. This leads to the accumulating of mutant p53 in the tumor cells at steady-state levels that greatly exceed those of wild type p53 in noncancerous cells [294].

TP53 germline mutations are the underlying cause of the Li-Fraumeni Syndrome, a familial disease characterized by early onset tumors including sarcomas, breast cancers, brain tumors, and adrenal cortical carcinomas[240, 241, 295]. The distribution of germline mutations is similar to somatic mutations, with mostly missense mutations (77%) located at the same hotspots [293].

Emergence of a *TP53* mutation within a cell might have three, not mutually exclusive, types of outcome. First, such mutation could abrogate the tumor suppressor function of the affected *TP53* allele, reducing the overall capacity of the cell to generate a proper p53 response; if the remaining allele is lost, such cells will be totally deprived of p53 wild type functions. Second, many mutant p53 isoforms can exert dominant–negative effects

over wild type p53 (p53^{wt}), by forming mixed tetramers that are incapable of DNA binding and transactivation. Hence, even if one wt allele is retained, the cell may become practically devoid of p53^{wt} function, particularly if the mutant protein is expressed in excess over its wt counterpart. Third, the emergent mutant p53 protein might possess activities of its own that were not present in the original p53^{wt} protein (gain of function) and which can actively contribute to tumor progression [296].

IV.5.1. Mutant p53 gain of function

Many *in vitro* studies have now shown the gain of function of mutant p53 (p53^{mut}). Human p53^{mut} was found to increase genomic instability by disrupting normal spindle checkpoint control, leading to accumulation of polyploid cells [297]. Several lines of evidence have confirmed this link between mutp53 and increased genomic instability, which is reflected by higher mutation rates, increased frequency of centrosome amplification, aberrant mitoses, increased gene amplification, and chromosome translocations [296].

More recent work using *in vitro* assays indicates that p53^{mut} can also augment cell migration and invasion [298-300]. Induction of cell migration by p53^{mut} is highly cell-context-dependent, and additional signals such as oncogenic Ras in combination with TGF-β might be required to unleash this activity [298].

The majority of tumor-associated p53 mutations occur within the DNA binding domain of p53. Thus, it is known that some mutant isoforms of p53 acquire novel transcriptional activities that contribute to the enhanced malignant potential of cells. For instance, it has been shown that p53^{mut} can up-regulate genes encoding many pro-proliferative or anti-apoptotic

proteins such as NFKB2, encoding the p52 subunit of NF-kB, cyclin A, cyclin B1, CDK1, CDC25C and hTERT [296].

On the other hand, many gain of function effects of p53^{mut} rely on its ability to bind and inactivate the p53-related proteins p63 and p73, a capacity that is absent in p53^{wt} [301]. At least some cancer-associated p53^{mut} proteins can engage in direct protein–protein interactions with some p73 and p63 isoforms, rendering them transcriptionally inactive. Reduction in p63 and p73 transcriptional activity has been shown to impair cell cycle arrest and apoptosis, thus promoting cell proliferation [302]. Recent reports have revealed a role for p63 in the metastatic properties of mutant p53-expressing cells. In these experimental systems, p63 had a negative effect on cell migration and invasion that was relieved upon its inactivation by p53^{mut} [298, 299]. Regarding p73, it has been described to be able to replace p53 in maintaining genome stability by suppressing aneuploidy and polyploidy in p53-deficient cells. Abrogation of p73 function by excess of p53^{mut} is thus, likely to augment the accumulation of cells with polyploidy genomes and aberrant chromosome numbers, thereby facilitating cancer progression [296].

IV.5.2 Mouse models of mutant p53

The first mutant p53 mouse models generated were those with deletions in the protein, that allowed to determine the consequences of loss of p53 wild type function. p53$^{-/-}$ mice are developmentally viable, but are prone to the spontaneous development of tumors; heterozygous mice also develop tumors, although at a later stage. p53$^{-/-}$ and p53$^{+/-}$ mice mainly present T-cell lymphomas and sarcomas, and tumors in these mice rarely metastasize[242, 303, 304].

The generation of mutant p53 knockin mice, was perhaps the most significant advance in the study of gain of function of p53. In these mice,

the endogenous $p53^{wt}$ allele was replaced by mutant versions, $p53^{R172H}$ and $p53^{R270H}$, which respectively mimic the common human hotspot mutations $p53^{R175H}$ and $p53^{R273H}$. These two studies showed that, when compared with $p53^{-/-}$ animals, mice carrying $p53^{mut}$ alleles tended to develop more aggressive, metastatic tumors. Furthermore, $p53^{mut}$ mice have a different tumour spectrum exhibiting a wider array of tumors, including an increased number of carcinomas and B-cell lymphomas, tumor types that are associated with p53 mutations in human cancer [305, 306].

In a skin carcinogenesis model driven by oncogenic KRas, mutant $p53^{R172H}$ mice exhibited increased tumor formation, accelerated tumor progression and elevated rates of metastasis, relative to their p53-null counterparts [307]. Very similar effects of combining endogenous $p53^{R172H}$ with endogenous oncogenic KRas were found in a mouse model of PDAC, where this combination led to a highly invasive, widely metastatic disease [192]. These results were confirmed by recent work using a similar PDAC mouse model, were the direct comparison of $p53^{R172H}$ with $p53^{flox}$ animals revealed that, despite similar kinetics of primary tumor formation, mutant $p53^{R172H}$ specifically promotes metastasis [308].

An opposite approach has also been used to study the effect of an increased level of p53 function. Mutant mice that express a truncated, constitutively activated form of p53 exhibited enhanced resistance to spontaneous tumours but displayed an early ageing phenotype[309]. A second model was developed which possessed an additional genomic copy of *Trp53*, retaining the normal regulation of p53. The “super p53” mice presented a robust enhancement of p53-mediated responses, exhibiting an increased DNA damage response and increased resistance to tumor development, with no evidences of premature aging [310].

IV.6. p53 in development and cell differentiation

IV.6.1. p53 and embryonic development

Tumor suppressive functions of p53 such as the regulation of cell proliferation and apoptosis are also tightly associated with the regulation of normal embryonic development [311].

To investigate the regulation of p53 transcription during mouse embryogenesis, several transgenic mouse models expressing a reporter gene under the control of the p53 promoter were generated [312-314]. These models demonstrated a differential expression pattern of p53: while in early embryos strong reporter activity was observed in most tissues, at later developmental stages, the activity became heterogenic and restricted to specific tissues, at distinct differentiation stages.

Despite the tight regulation of p53 and its known fundamental roles, the apparently normal phenotype of p53-null mice at birth[242] strongly suggested that p53 is dispensable for proper development. However, it has subsequently become clear that p53-null mice exhibit a significant frequency of developmental defects. This is most clearly manifested as a reduction in p53-null female progeny, which tend to develop exencephaly[315]. In addition, other developmental anomalies are present, including ocular abnormalities, polydactily of the hind limbs, defects in upper incisor tooth formation, and a lower fertility of both males and females [316-318]. The fact that p53 knockout mice do develop and are born alive indicates that there is an incomplete penetrance of the p53-null phenotype, suggesting the existence of compensatory mechanisms. This might be explained by the fact that the other p53 family members, p63 and p73, which are expressed during mouse embryogenesis, may compensate for the absence of p53 [311]. The importance of p53 during development

seems more pronounced in other species such as Xenopus, where p53 expression is essential for normal development [319].

The role of p53 in development was also demonstrated in mouse models that exhibit increased p53 protein levels due to disruption of the Mdm2 or MdmX genes. Both Mdm2 and MdmX knockout mice die during early embryogenesis due to a failure of p53 inhibition during gestation that results in accelerated cell-cycle arrest and apoptosis during a stage in which rapid cell divisions are required [320, 321]. The direct contribution of p53 to this phenotype was evident by concomitant deletion of p53, which completely rescued the embryonic lethality of Mdm2 and MdmX-deficient mice [322-324]. Thus, the tight regulation of p53 levels and its activity along distinct developmental stages is required for proper development.

There is also evidence for an essential role of the other members of the p53 family, p63 and p73, in the regulation of differentiation and development. DNp63 and DNp73 isoforms have been shown to support self-renewal of stem cells, while full-length isoforms of p53, p63 and p73 induce differentiation [257], by which the balance of various p53 family isoforms may determine cell fate. p63 was found to have an important role in embryonic development, being required for the morphogenesis of epidermis and its derivatives and for limb development [325, 326], while p73 was shown to be essential essential for neuronal development and differentiation [327, 328]. Thus, the whole p53 family network emerges as an important regulator in embryonic development and this may also point at its possible involvement in the differentiation programs that occur in adult tissues.

IV.6.2. p53 and stem cells

p53 plays an important role in maintaining genomic stability of embryonic stem cells (ESCs) by coordinating both their self-renewal capacity and DNA

damage responses [329]. ESCs have been shown to lack the induction of a cell cycle G1/S checkpoint or cellular senescence after DNA damage [330, 331]. In these cells activation of p53 after DNA-damage has been shown to induce the differentiation of ESCs into other cell types, which could efficiently undergo p53-dependent cell cycle arrest or apoptosis. p53 induces differentiation in ESCs by directly suppressing the expression of Nanog which is the master transcriptional factor that maintains the self renewal of ESCs [332]. Recent establishment of human $p53^{-/-}$ ESCs revealed that the role of p53 in suppressing pluripotency is conserved in mouse and human [333]. Germ cells can also be spontaneously reprogrammed to pluripotent stem cells in the absence of p53, further supporting a potential role of p53 in suppressing dedifferentiation [334].

A role of p53 in pluripotency has recently been shown through studies of cellular reprogramming. The nucleus of an adult somatic cell can be reprogrammed into a pluripotent state [335]. Yamanaka and colleagues identified a panel of transcription factors (Oct4, Sox2, Klf4 and c-Myc) that are sufficient to reprogram mouse somatic cells into ‘induced pluripotent stem cells’ (iPSCs) [336]. OCT4 and SOX2 are required for reprogramming, but the combination of other factors can be flexible, as different cocktails can also reprogram human somatic cells into iPSCs [337]. The frequency of reprogramming of primary cells is very low even when all reprogramming factors are expressed, indicating that there are barriers that block successful reprogramming. All reprogramming factors have some oncogenic potential and their overexpression in somatic cells induces oncogenic stress, resulting in activation of p53-dependent pathways [329]. In fact, a series of recent papers reported that functional ARF and p53 constitute an effective barrier for induced pluripotency. By comparing the reprogramming efficiency of WT and p53-deficient mouse and human

fibroblasts, several studies have shown a strong increase in the reprogramming frequency of p53-deficient cells [338-341]. The link between oncogenic signaling and the suppression of reprogramming is supported by the findings that the elimination of ARF, an activator of p53 in response to oncogenic stress, also greatly increases reprogramming efficiency [342].

A number of recent compelling publications has revealed an important role of p53 in adult multipotent cell populations, namely in neural [343, 344], hematopoietic [345, 346] and mammary [347, 348] stem cells.

Loss of p53 provides an advantage to neural stem cells and/or early progenitor cells. p53 was found to be expressed at higher levels in cells of the neural stem cell niche and p53 deficient neural stem cells cultured as neurospheres presented an increased self-renewal capacity, increased cell proliferation, and a reduction in apoptosis [343]. Similarly, in olfactory bulb neural stem and progenitor cells cultured as neurospheres, loss of p53 promotes neurosphere formation and stem cell self-renewal [344].

p53 also plays an important role also in the hematopoietic system, where it has been shown to limit the expansion potential of multipotent progenitors together with Ink4a/Arf, [345] and to regulate hematopoietic stem cell quiescence [346].

In the breast epithelium, two independent studies revealed that mammary epithelial cells lacking p53 present increased mammosphere formation ability and enhanced capacity to reconstitute tissue upon transplantation[347, 348]. This function of p53 is related with the regulation of asymmetric stem cell division as, in its absence, mammary stem cells undergo an increased number of self-renewing divisions [347]. p53 was also shown to restrict the stem cell potential of mammary progenitor cells through activation of the Notch pathway [348].

V. Epithelial-mesenchymal transition

The epithelial-mesenchymal transition (EMT) is a biological process that allows an epithelial cell to undergo coordinated changes enabling it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, and increased production of ECM components [349].

EMT plays a crucial role in the formation of multiple tissues and organs during embryonic development and is also involved in wound healing, tissue regeneration, and organ fibrosis. Moreover, the EMT process has an important role in carcinogenesis, contributing to increased cellular invasion, metastatic dissemination and acquisition of therapeutic resistance [350, 351].

V.1. The regulation of the EMT process

Epithelial cells are closely attached to their neighbors through the sequential arrangement of adherens junctions, desmosomes, and tight junctions, displaying an apico-basal axis of polarity. The epithelial cell layer maintains global communication through gap junctional complexes, and it remains separated from adjacent tissues by a basal lamina. By contrast, mesenchymal cells are non-polarized and are loosely organized in a three-dimensional extracellular matrix (ECM) [351].

The EMT process involves dramatic changes in attachment structures, in the cytoskeleton, and in the ECM composition that act together to alter cell morphology. EMT-inducing signals lead to the disruption of intercellular adhesion structures and loss of the characteristic apico-basal polarity of the epithelial cells. E-cadherin is a paradigm target protein in these processes, but many other proteins involved in intercellular adhesion are also involved. Cytoskeletal changes initially occur by formation of apical

constrictions and disorganization of the basal cytoskeleton, being crucial for cells to leave the epithelium and begin migrating individually. Simultaneously, there's a dramatic reorganization of the ECM, through the upregulation of the expression of ECM proteins (such as fibronectin and collagens), proteases (such as MMPs) and other remodeling enzymes (such as lysyl-oxidase). Protease activity leads to breakdown of the basement membrane and cell ingression. Thus, upon EMT, mesenchymal cells acquire migratory and invasive properties that allow them to move through the extracellular matrix [349, 350, 352].

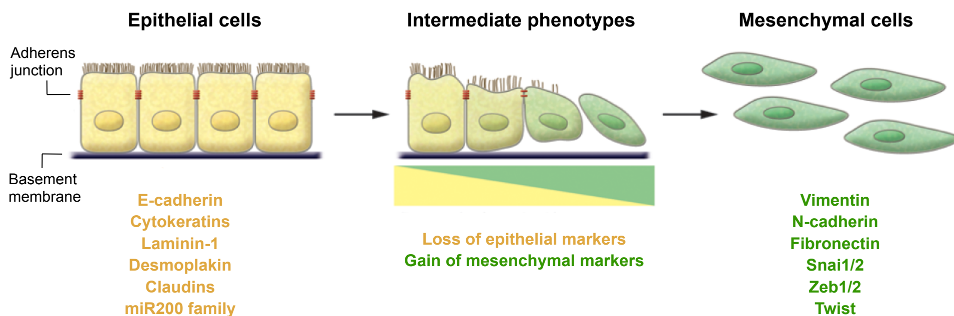


Figure I13 Epithelial-mesenchymal transition (EMT). EMT involves a functional transition of polarized epithelial cells into mobile and ECM component-secreting mesenchymal cells. The epithelial and mesenchymal cell markers commonly used by EMT researchers are listed. Colocalization of these two sets of distinct markers defines an intermediate phenotype of EMT, indicating cells that have passed only partly through an EMT. *Adapted from Kalluri and Weinberg, 2009* [349].

V.1.1. Mechanisms of EMT activation

Multiple extracellular signals can initiate an EMT program, and there is a significant crosstalk among the downstream intracellular signaling pathways and transcription factors that orchestrate this complex program, including multiple positive-feedback loops.

Members of the transforming growth factor-β (TGFβ) family of cytokines are the best characterized inducers of EMTs occurring during the course of embryonic development, wound healing, fibrotic diseases and cancer pathogenesis. TGFβ may induce EMT through multiple signaling mechanisms, including TGFβ receptors direct phosphorylation of SMAD transcription factors [169] and of proteins regulating cell polarity and tight junction formation, such as PAR6A [353]. TGFβ also influences the activities of multiple other EMT-inducing signal transduction pathways, including those involving Notch, Wnt and integrin signaling, some of which can act in concert to trigger EMT programmes [350, 354].

Wnt signalling can lead to EMT through the inhibition of the degradation of β-catenin degradation. The resulting increased levels of β-catenin enable this molecule to translocate to the nucleus where it can serve as a coactivator of TCF transcription factors, inducing the expression of several genes, including several EMT inducing transcription factors. Loss of E-cadherin expression also results in the liberation of β-catenin, which is normally sequestered by the cytoplasmic tail of E-cadherin at adherens junctions. Notch signalling can also induce an EMT by activating the nuclear factor-κB (NF-κB) pathway or by modulating the activity of TGF-β signaling. The NF-κB pathway activates EMT through induction of SNAI1 [351, 354].

Numerous receptor tyrosine kinases (RTKs), specially the Hepatocyte Growth Factor (HGF) receptor c-MET, have also been found to have crucial roles in embryonic processes that involve EMT programmes. Constitutive RTK activation together with E-cadherin loss can actually lead in many cancer cells to the stabilization of the mesenchymal state [350].

Hypoxia is a physiological mechanisms that can also induce EMT in tumours through multiple distinct mechanisms, including upregulation of hypoxia-

inducible factor-1α (HIF1α), hepatocyte growth factor (HGF), SNAI1 and TWIST1, activation of the Notch or NF-κB pathways, and induction of DNA hypomethylation [355].

V.1.2. Transcriptional control of EMT

One of the hallmarks of EMT is the functional loss of E-cadherin (encoded by the *CDH1* gene) and the characterization of E-cadherin transcriptional regulation has provided important insights into the molecular mechanisms implicated in EMT. Several transcription factors have been demonstrated to repress E-cadherin expression and to control EMT programs during embryonic development and in cancer. These include direct transcriptional repressors of E-cadherin expression, such as SNAIL/SNAI1, SLUG/SNAI2, ZEB1, SIP1/ZEB2 and E47/E2α, and those that act indirectly on E-cadherin, such as TWIST1, Goosecoid (GSC) and FOXC2 [351, 356].

SNAI1 and SNAI2 belong to the Snail superfamily of zinc finger transcriptional repressors. These proteins bind to E-box consensus sequences in the *CDH1* promoter with the help of local modifications of chromatin structure after the recruitment of SIN3A, histone deacetylases HDAC1 and HDAC2, and components of the Polycomb 2 complex [356].

ZEB1 and ZEB2 are members of the ZEB family and interact with DNA through the simultaneous binding of the two zinc-finger domains to high-affinity binding sites composed of bipartite E-boxes, as found in the *CDH1* promoter. ZEB factors act through the recruitment of either co-activators (PCAF or p300 for ZEB1) or co-repressors (CTBP for ZEB2) [356].

TWIST1 and GSC also downregulate E-cadherin expression, albeit indirectly. FOXC2, which lies downstream of TWIST1, GSC and SNAI1 does not affect E-

cadherin expression, but rather promotes its cytoplasmic localization. Usually the indirect EMT inducers activate some of the direct repressors[354].

Emerging data suggest extensive crosstalk among these transcription factors, allowing them to form a signaling network that is responsible for establishing and maintaining mesenchymal cell phenotypes. In addition to their function as E-cadherin repressors, these transcription factors have also been shown to repress epithelial cell polarity and cell division, while promoting cell survival. In a tumorigenic context, this would favor invasion versus tumor growth, and the resistance to cell death would also confer a selective advantage on cancer invasive cells to populate distant organs[351].

V.1.3. Non-coding RNAs as regulators of EMT

Recent studies have also highlighted the importance of microRNAs (miRNAs) in the regulation of the epithelial phenotype, by controlling the expression of the EMT inducers. For instance, microRNAs of the miR-200 and miR-205 families have been shown to regulate the expression of ZEB factors and to control the invasive and metastatic potential of cancer cells[357-359]. The miR-200 family includes the miRNAs miR-200a, miR-200b, miR-200c, miR-141 and miR-429, which all have been seen to regulate the expression of both ZEB1 and ZEB2 proteins [360]. On the other hand, ZEB1 can suppress the expression of miR-200 and miR-141, generating a feedback loop that controls the establishment of the mesenchymal phenotype [361]. This finding was extended to all miR-200 family members [362], revealing that Zeb factors and miR-200 form a double-negative feedback loop, reciprocally controlling the expression of each other. Consequently, an emerging idea is that micro-RNAs and EMT inducers form a regulatory cascade that regulates the epithelial/mesenchymal phenotype, not only during carcinogenesis, but also during development [351].

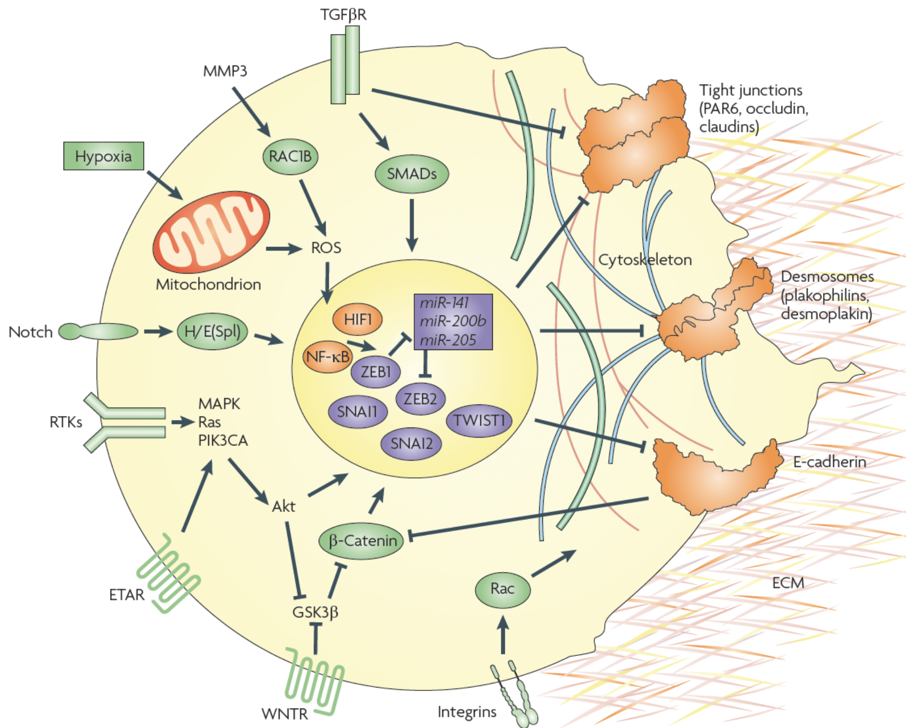


Figure I14. Signaling networks regulating EMT. Receptor tyrosine kinases (RTKs), transforming growth factor-β (TGFβ), Notch, endothelin A receptor (ETAR), integrins, Wnt, hypoxia and matrix metalloproteinases (MMPs) can induce EMT through multiple signaling pathways. EMT is associated with dramatic changes in the cytoskeleton and extracellular matrix (ECM) composition and attachment that act together to alter cell morphology. EMT-inducing signals can lead to the disruption of tight junctions, desmosomes and adherens junctions and result in the dramatic reorganization of the ECM as many EMT-inducing factors upregulate the expression of ECM proteins (such as fibronectin and collagens), proteases (such as MMPs) and other remodeling enzymes (such as lysyl oxidase). Besides the interaction among the various signaling pathways, there is also extensive crosstalk among the EMT-inducing transcription factors (purple circles) and the microRNAs (miRNAs) regulating them. *From Polyak and Weinberg, 2009* [350].

V.2. EMT in development and injury

During embryonic development, both epithelial-mesenchymal transition (EMT) and the reverse process, mesenchymal-epithelial transition (MET), are necessary for the differentiation of specialized cell-types and the development of most adult tissues and organs. Primary EMT events take place during implantation of the embryo into the uterus, during gastrulation, and during neural crest formation in amniotes. In the embryo, the first EMT event occurs at gastrulation, where a subset of cells from the epiblast, the single epithelial cell layer of the embryo, move to the midline to form the primitive streak. These cells undergo EMT and internalize to generate mesoderm and endoderm, while those remaining in the epiblast become ectoderm. Mesoderm and endoderm contribute to many tissues of the adult organism that themselves undergo several rounds of EMT and MET. For example, paraxial mesoderm undergoes MET to form the somites and endodermal derivatives can also use EMT to generate internal organs, such as the pancreas and liver. Some cell lineages can undergo several cycles of EMT, as occurs with the heart, which forms through three successive cycles of EMT and MET [352].

A process similar to EMT occurs also as a physiologic response to injury. During wound healing, keratinocytes at the border of the wound acquire an intermediate phenotype that allows them to move while maintaining loose contacts rather then migrating as individual cells [351]. EMT also occurs in pathologic conditions that involve organ degeneration such as fibrosis. Fibrosis was originally thought to originate exclusively through the activation of interstitial fibroblasts that convert to myofibroblasts. However, lineage tracing studies have shown that, in renal fibrosis, many of these myofibroblasts come from epithelial cells of the renal ducts that

convert to mesenchymal cells through an EMT process [363]. More recently, similar studies have shown that an EMT process can also occur in lens epithelium, endothelium, hepatocytes and cardiomyocytes, contributing to tissue fibrosis[351].

V.3. EMT in cancer

The EMT program was first described as a cell culture phenomenon and its significance to *in vivo* carcinogenesis was long debated. However, evidence has accumulated about the relevance of this process in human tumors and experimental mouse models of cancer [350].

Several genes involved in regulating EMT programs are altered in tumors owing to regulatory, genetic, and epigenetic events. Germline mutations in *CDH1* have been identified as the cause of hereditary diffuse gastric carcinomas [364] and somatic inactivation of this gene occurs very frequently in lobular breast carcinomas [365]. Promoter hypermethylation is also an important epigenetic event associated with loss of E-cadherin gene expression during cancer progression, and is related with a more invasive and metastatic phenotype in breast cancer cells [366].

In solid tumors, EMT was found to occur at the invasive front of the tumor, producing single migratory cells. This has been observed in colon, breast, cervical and papillary thyroid carcinomas. Furthermore, increased expression of EMT inducers such as SNAI1 and SNAI2, has been shown to be associated with disease relapse and survival in patients with breast, colorectal and ovarian carcinoma, indicating that an EMT gene signature is correlated with poor clinical outcome. EMT has also been related with the acquisition of resistance to chemotherapy in cells of colon, ovarian and breast carcinomas [351].

Similar to their role in embryonic development, both EMT and MET seem to have crucial roles in the tumorigenesis. In particular, EMT has been found to contribute to invasion and metastatic dissemination while MET occurs following dissemination during the establishment of distant metastases [350].

V.3.1. EMT and PDAC

Regarding pancreatic ductal adenocarcinoma, studies revealed a reduction or loss of E-cadherin expression in 42-53% of human samples and loss of E-cadherin was correlated with lack of differentiation and with lymph node and distant metastasis [367, 368]. Several signaling pathways have been shown to induce EMT and invasion in PDAC cultured cells, including TGF-β [369], HGF[370], BMPs[371] and VEGF [372]. Recently, a study using an *in vivo* model for selection of metastatic pancreatic cancer cells has revealed that highly metastatic cells have a more mesenchymal phenotype. In these cells, silencing of E-cadherin has been shown to be mediated by a complex containing Snail and histone deacetylases (HDAC) 1 and 2 [373]. Furthermore, several studies have demonstrated that pancreatic cancer resistance to chemotherapy is associated with the acquisition of EMT traits [374-376]. The mechanism allowing the resistance seems to be dependent of ZEB1 expression [374], and is linked with activation of the Notch pathway [376] and with downregulation of pro-epithelial miRNAs, such as miR-200 and let-7[375].

V.3.2. EMT and cancer stem cells

EMT has been implicated in two of the most important processes responsible for cancer-related mortality: progression to distant metastatic disease and acquisition of therapeutic resistance. Both of these processes may be linked, in turn, to a third: the generation by EMT of cancer cells with stem cell-like characteristics [350].

Two independent studies have demonstrated that, induction of EMT in mammary epithelial cancer cells, generated cells with stem cell properties[377, 378]. Furthermore, breast cancer cells with stem cell characteristics and expressing EMT-associated genes have been found to be increased in residual tumors after chemotherapeutic treatment [379].

Recent studies have also reinforced the connection between EMT and the acquisition of stemness properties. TWIST, an important mediator of EMT regulates the expression of BMI1 and that both proteins are required for the EMT and the stemness in head and neck squamous cell carcinoma [380]. Likewise, ZEB1 has also been shown to increase stem-cell properties and tumorigenic capacity in pancreatic cancer cells. ZEB1 acts by repressing the miRNAs miR-200, miR-183 and miR-203, which target the stemness factor BMI1 and possibly KLF4 and SOX2 [381]. Reciprocally, the mir200 family also targets ZEB EMT regulators, constituting a feedback loop that controls both EMT and stemness and which is thought to be involved in the regulation of the differentiation state of cancer stem cells [360].

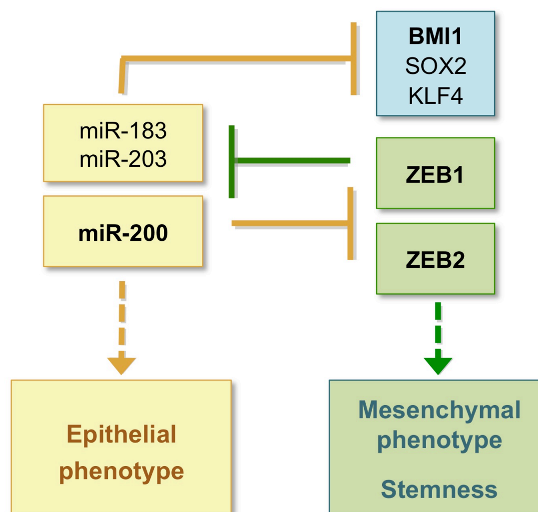


Figure I15. The ZEB/miR-200 negative feedback loop. miR-200 family members inhibit the expression of ZEB transcription factors, promoting an epithelial phenotype. ZEB1 suppresses the expression of the mir-200 family, as well as miR-183 and miR-203, which together inhibit the expression of stem cell factors, thus linking the induction of EMT with the acquisition of stem cell traits. *Adapted from Brabletz and Brabletz, 2010* [360].

HYPOTHESIS & AIMS

Hypothesis

Pancreatic acinar cells play an important role in pancreatic diseases such as chronic pancreatitis and PDAC. The development of conditions to culture acinar cells should allow the analysis of their differentiation potential. The use of murine pancreas provides the opportunity to assess the role of key proteins involved in pancreatic acinar cell differentiation using genetic tools. These studies should provide clues as to the mechanisms implicated in the development of chronic pancreatitis and PDAC.

Aims

The general goal of this project was to study pancreatic acinar cell plasticity and the mechanisms involved therein, with the purpose of providing a greater knowledge regarding the pathophysiology of chronic pancreatitis and PDAC.

The specific objectives of the study were:

1. To establish an *in vitro* model for the culture of primary murine pancreatic acinar cells and to characterize the phenotype of the cultures.

2. To analyze the mechanisms involved in the plasticity of the cultured acinar cells.

3. To determine the relevance of the *in vitro* model to cellular events associated with the response to the experimental induction of pancreatitis.

4. To assess the role of specific genes in the regulation of acinar cell growth and differentiation using genetically modified mice.

RESULTS

I. Research Publication 1

Adult pancreatic acinar cells dedifferentiate to an embryonic progenitor phenotype with concomitant activation of a senescence programme that is present in chronic pancreatitis

Pinho AV, Rooman I, Reichert M, De Medts N, Bouwens L, Rustgi AK, Real FX. Adult pancreatic acinar cells dedifferentiate to an embryonic progenitor phenotype with concomitant activation of a senescence programme that is present in chronic pancreatitis. Gut, 2010; In press.

Pinho AV, Rooman I, Reichert M, De Medts N, Bouwens L, Rustgi AK, et al. Adult pancreatic acinar cells dedifferentiate to an embryonic progenitor phenotype with concomitant activation of a senescence programme that is present in chronic pancreatitis. Gut. 2011; 60 (7): 958-66.

I.1. Supplementary Material

I.1.1. Supplementary materials and methods

Cell isolation and culture for lineage tracing experiments and X-gal staining. For lineage tracing, we isolated acini from 8-10 week old Elastase-CreERT2 ($Ela^{CreERT2}$); R26R-LSL-LacZ mice (from D. Stoffers, University of Pennsylvania) that were pretreated with Tamoxifen (T5648, Sigma St.Louis, Mo, USA) dissolved in 10% ethanol/90% NaCl, given orally by gavage in three doses (20mg, 20mg and 10mg) over one week, as described previously[1]. At least one week elapsed before isolating the cells. Acinar cell labeling ranged between 20 and 50% (n=8). A variable leakage of the construct (0.5-30%)(n=6) in amylase-positive acinar cells - but not in Krt19 positive duct cells - was noted in the absence of tamoxifen administration. Labeling of some centroacinar cells cannot be excluded. Cells were cultured as described in Materials and Methods; soybean trypsin inhibitor was added for 2 days (0.1 mg/mL, Sigma). For adherent conditions, cells were cultured on tissue-culture treated 24-well plates. For X-gal staining, cells were fixed with 4% paraformaldehyde (Merck) for 15 min on ice and washed with PBS. Aggregates were embedded in OCT, snap frozen in liquid nitrogen, and sectioned. X-gal permeabilization buffer ($MgCl_2$, DOC, NP40) was added for 15 min at 4 °C. X-gal detection buffer (Tris, $MgCl_2$, $C_6FeK_3N_6$ and $C_6FeK_4N_6.3H_2$, ultrapure X-gal, DMSO) pH 7.4 was added overnight at 37°C.

RT-PCR. Total RNA was isolated using the GenElute Mammalian Total RNA kit (Sigma) and RNA integrity was assessed using an Agilent 2100 Bioanalyzer. Following DNAse treatment (DNAfree, Ambion), cDNA was prepared from total RNA (Taqman Reverse Transcription Reagents kit,

Applied Biosystems) and 20 ng RNA equivalent was used for PCR with specific primers (**Supplementary Table 3**) in the presence of SYBR GreenER (Invitrogen) using the 7900H Fast Real Time PCR System (Applied Biosystems). All analyses were done in duplicate. A melting curve analysis was performed for each reaction to control for product quality and specificity; selected PCR products were sequenced. The expression levels were normalized to individual Hprt expression.

Chromatin immunoprecipitation. The protocol was followed as outlined in www.upstate.com/ChIP. Acinar cells were cultured in suspension for 5-6 days and acini were freshly isolated on the day of ChIP analysis. From both samples, chromatin was prepared after formaldehyde cross-linking, extraction in SDS lysis buffer, and sonication to DNA fragments of 200-800 bp. Samples were diluted in ChIP dilution buffer and immunoprecipitated with the relevant antibodies, or a non-immune serum of the same species, and with pre-blocked protein G-Sepharose (Calbiochem). The beads were washed, cross-linking was reversed, and the DNA was extracted from the solution (Illustra GFX PCR DNA, GE Healthcare). The DNA was quantified by real-time PCR analysis. Primers are listed in **Supplementary Table 4**. The following antibodies were used: rabbit anti-Ptf1a (our own laboratories), rabbit anti-Rbpjl (R. Wagener, University of Köln, Germany), and goat anti-Rbpj (SC-8213, Santa Cruz Biotechnology).

Immunocytochemistry. Cell pellets and tissues were fixed in buffered 4% formaldehyde and processed for paraffin embedding, or fixed with cold 4% paraformaldehyde, preincubated in 30% sucrose, and embedded in OCT. Monolayer-cultured cells were stained directly in the culture wells. Permeabilisation of the cells in the monolayers was achieved by incubation in methanol at -20 °C. For all immunocyto/histochemistry, Avidin Biotin

Complex-method was applied when using peroxidase and diaminobenzidine as substrate (Dako, Glostrup, Denmark). For immunofluorescence, secondary antibodies directly coupled to a fluorochrome were applied with or without TSA (Perkin Elmer, Boston) amplification. Antigen retrieval on paraffin sections was performed by microwave/steam heater boiling in citrate buffer (pH 6). Histological images were acquired with a Nikon TE2000E microscope using NIS AR2.30. Immunofluorescence images were also acquired on a confocal ultra-spectral microscope Leica TSC-SP2_AOBS-UV. The following antibodies were used: rabbit anti-amylase (A8273, Sigma), mouse anti-carboxypeptidase A (C2163, Sigma), rabbit anti-chymotrypsin (our own laboratories), rabbit anti-poly-keratin (Z0622, DAKO), rat anti-keratin 19 (Troma III, Developmental Studies Hybridoma Bank), rabbit anti-Ptf1a (B. Bréant, U. Paris VI, Paris, France), rabbit anti-Pdx1 (A. Skoudy, IMIM, Barcelona, Spain), rabbit anti-Hes1 (T. Sudo, Toray Industries Inc., Japan), rabbit anti-Foxa1 (ab23738, Abcam), rabbit anti-Sox9 (ab5535, Chemicon), goat anti-Sca1 (BAF1226, R&D Systems), mouse anti-Cd133 (ab19898, Abcam), rabbit anti-Aldh1 (ab23375, Abcam), rabbit anti-p53 (NCL-p53-CM5p, Leica Biosystems Newcastle, Lda), rabbit anti-Ki67 (NCL-Ki67p, Leica Biosystems Newcastle, Lda), mouse anti-BrdU (G3G4, Developmental Studies Hybridoma Bank), rabbit anti-Caspase 3 Active (AF835, R&D Systems), rabbit anti-Dec1 (A.L. Harris, Oxford, U.K.), mouse anti-phospho-histone H2AX (05-636, Millipore), rabbit anti-53BP1 (NBP1-19447**,** Novus Biologicals); goat anti-Hnf1β, rabbit anti-p16/Ink4a, goat anti-p21, goat anti-Foxa2, and rat anti-p19/Arf were purchased from Santa Cruz Biotechnology (respectively SC-7411, SC-1207, SC-397-G, SC-9187 and SC-32748).

Transfections. HEK-293T cells were transfected with pSG5-Rbpjl or pSG5-Rbpj plasmids [kind gifts of R. J. MacDonald (University of Texas

Southwestern, Dallas, TX) and A. Bigas (IMIM, Barcelona), respectively] using Fugene 6 (Roche Diagnostics) following manufacturer's instructions. Cellular lysates were obtained 48h after transfection.

Western Blotting. Proteins from HEK-293T cells were extracted in lysis buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA pH 8.0, 0.5% NP-40) supplemented with protease inhibitors (Complete, EDTA-free protease inhibitor cocktail, Roche Diagnostics). Proteins from pancreatic tissue and primary cultured cells were extracted in Laemmli buffer (10% glycerol, 2.3% SDS, 0.125 M Tris-HCl pH 6.8) supplemented with the same protease inhibitors and phosphatase inhibitor cocktail 3 (Sigma). Proteins were separated on SDS-polyacrylamide gels and blotted onto a nitrocellulose membrane. The following antibodies were used: rabbit anti-Rbpl (R. Wagener), rabbit anti-Dec1 (A. L. Harris), mouse anti-β-actin (A5441, Sigma), and mouse anti-Ras (clone Ras10, 05-516, Upstate Biotechnology). Goat anti-Rbpj (SC-8213), rabbit anti-p53 (SC-6243), goat anti-p21 (SC-397-G), and goat anti-Akt1/2 (SC-1619) were from Santa Cruz Biotechnology. Rabbit anti-phospho-p42/p44 MAPK (#9101), rabbit anti-phospho-Akt (#9271), rabbit anti-p42/p44 MAPK (#9102), and rabbit anti-Caspase 3 (#9662) were from Cell Signaling Technology.

BrdU proliferation analysis. BrdU incorporation of isolated acini samples was performed by intraperitoneal injection of 50mg/kg of BrdU (Sigma) in saline, administered 24h prior to isolation of acinar cells. BrdU incorporation of D5 suspension and monolayer cultures was performed *in vitro* by adding BrdU (Sigma) to the culture medium (50μM) 24h prior to cell fixation. Immunocyto/histochemistry was performed as described previously.

Senescence-associated β-galactosidase staining. Total pancreas samples and cell pellets were embedded in OCT and frozen; staining was performed on cryosections. For monolayer cultures, staining was performed directly in the culture well. Senescence asoociated β-galactosidase staining was performed as described by Dimri et al[2], using a commercial kit (Cell Signaling Technology) and following the manufacturer's instructions.

Ras activation assay. Activated Ras was detected using a Ras Activation Assay Kit (Upstate Biotechnology) according to manufacturer's procedure. Briefly, total pancreas, isolated acini, 24h, and D5 cultured acini were lysed in Mg2+ lysis buffer and 1 mg of protein was incubated with Raf-1 Ras-binding domain fused to a glutathione S-transferase (GST-Raf1-RBD) agarose conjugate. Precipitates were washed 3 times, resuspended in Laemmli buffer, and boiled for 5 minutes. Proteins were separated using SDS-polyacrylamide gels, blotted onto a nitrocellulose membrane, and subjected to immunoblot analysis using mouse anti-RAS antibody. Input (10% of total used for assay) was analyzed in parallel and immunoblotted against anti-Ras and anti-β-actin.

Expression arrays and data analysis. The acinar fraction and D5 suspension cultures were established as indicated above; normal pancreatic ductal cells were isolated from fresh mouse pancreas (Reichert M, Rustgi AK, manuscript in preparation). Total RNA from three independent experiments was extracted using RNaqueous kit (Ambion) according to manufacturer's instructions and RNA quality was determined using the Agilent 2100 bioanalyzer. mRNA expression analysis was performed using the MoGene-1_0-st-v1 platform from Affymetrix and raw expression data was extracted using the Expression Console software. Differential expression analysis was performed using the web-based suite, Pomelo II[3], a limma paired t-test was

used to determine significantly differentially regulated genes between D5 culture samples and isolated acini and a limma t-test was used to determine significantly differentially regulated genes between these two samples and isolated ducts. A false discovery rate FDR of less than 0.01 and a fold-change>2 was used to assign significantly differentially regulated genes. For selected comparisons, an FDR threshold of 0.05 was used. Gene Set Analysis (GSEA)[4] was carried out using Biocarta pathway annotation. Acinar and ductal specific genes relevant to pancreas pathology were also selected from the array. The microarray data were used for principal component analysis.

Densitometric quantification. For relative quantification, band intensities were analyzed using Quantity One from Bio-Rad Laboratories. Relative levels of activated Ras, total Ras and phosphorilated proteins were determined using β-actin as reference since important changes in total protein were observed during culture.

Statistical analyses. *In vitro* data were analyzed by two-tailed Student t-test or Mann Whitney, statistical significance was accepted at a confidence interval <0.05 (GraphPad Prism). Mean values are given ± SEM. The number of independent experiments is indicated in the text as n.

References

1. Solar M, Cardalda C, Houbracken I, et al. Pancreatic exocrine duct cells give rise to insulin-producing beta cells during embryogenesis but not after birth. Dev Cell 2009; 17:849-60.

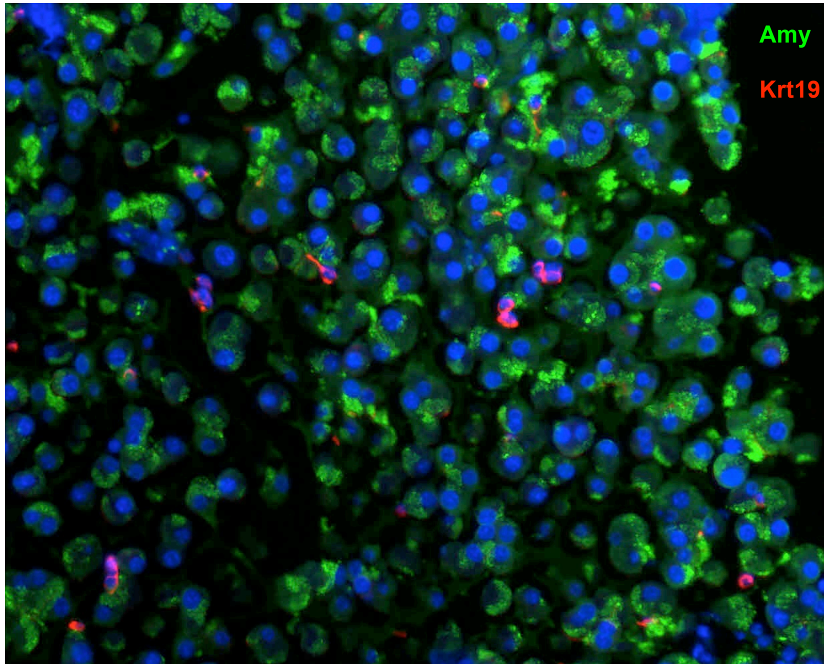
2. Dimri GP, Lee X, Basile G, Acosta M, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 1995; 92:9363-9367.

3. Morrissey E, Diaz-Uriarte R. Pomelo II: finding differentially expressed genes. Nucleic Acids Research 2009; 37:W581-W586.

4. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA 2005; 102:15545-15550.

I.1.2. Supplementary Figures

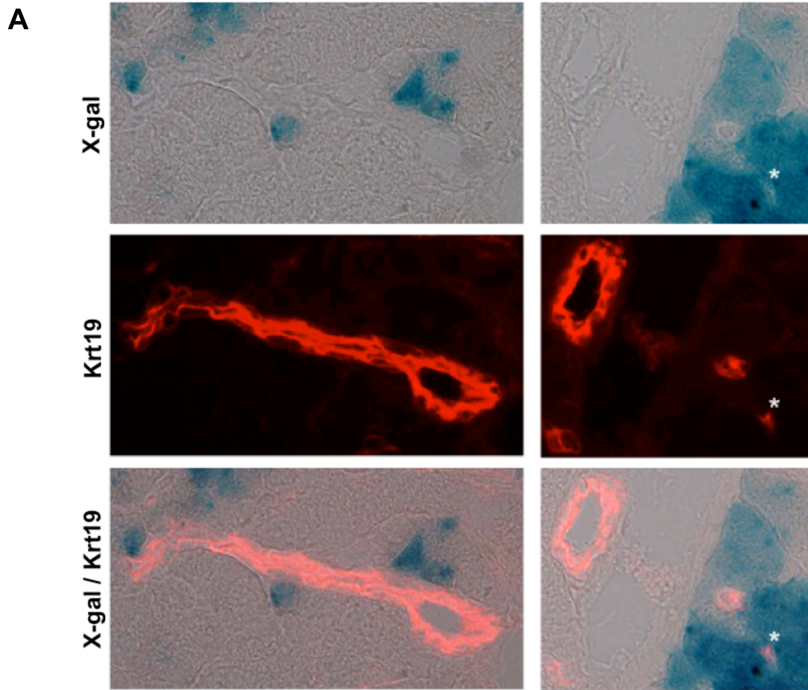
A

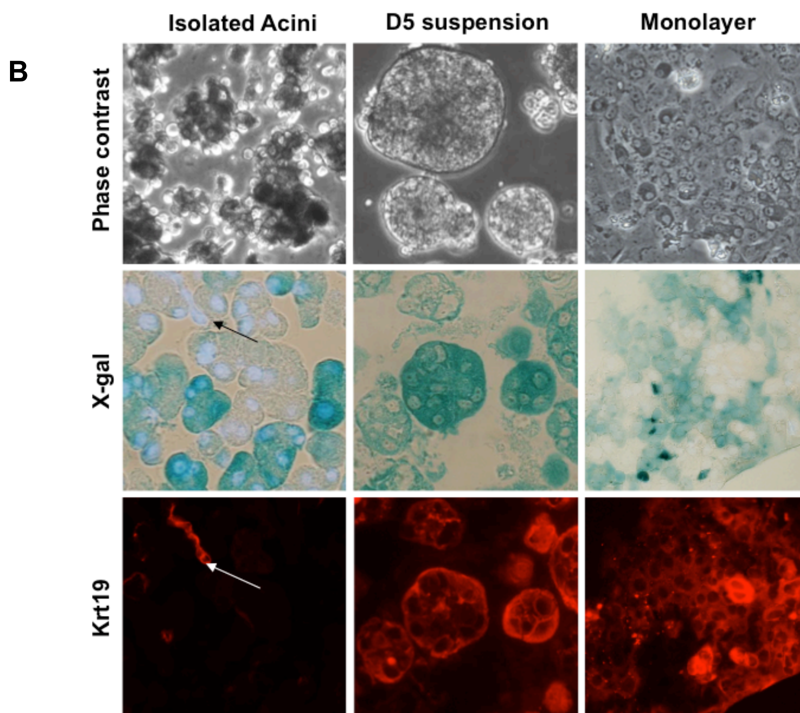


B

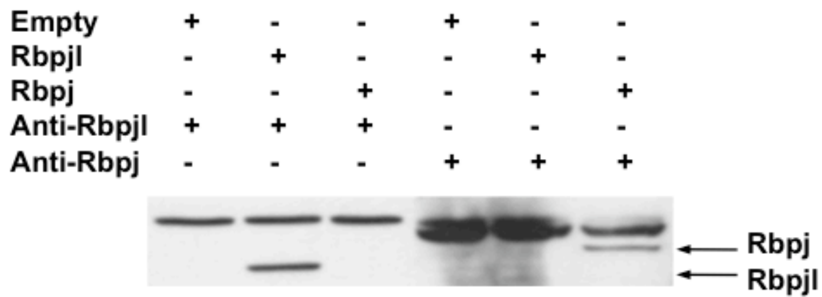
	Mean ± SEM
Amy +	92.3 ± 1.1 %
Krt19 +	3.8 ± 0.9 %
Amy - / Krt19 -	3.5 ± 0.4 %

Supplementary Figure 1. Characterization of the isolated acinar fraction. A. Double immunofluorescence for the acinar enzyme amylase and the ductal marker Krt19 in isolated acini. Nuclei were counterstained with DAPI. **B.** Quantification of the positive and negative cells for amylase and Krt19. Results represent an average of 6 independent experiments.

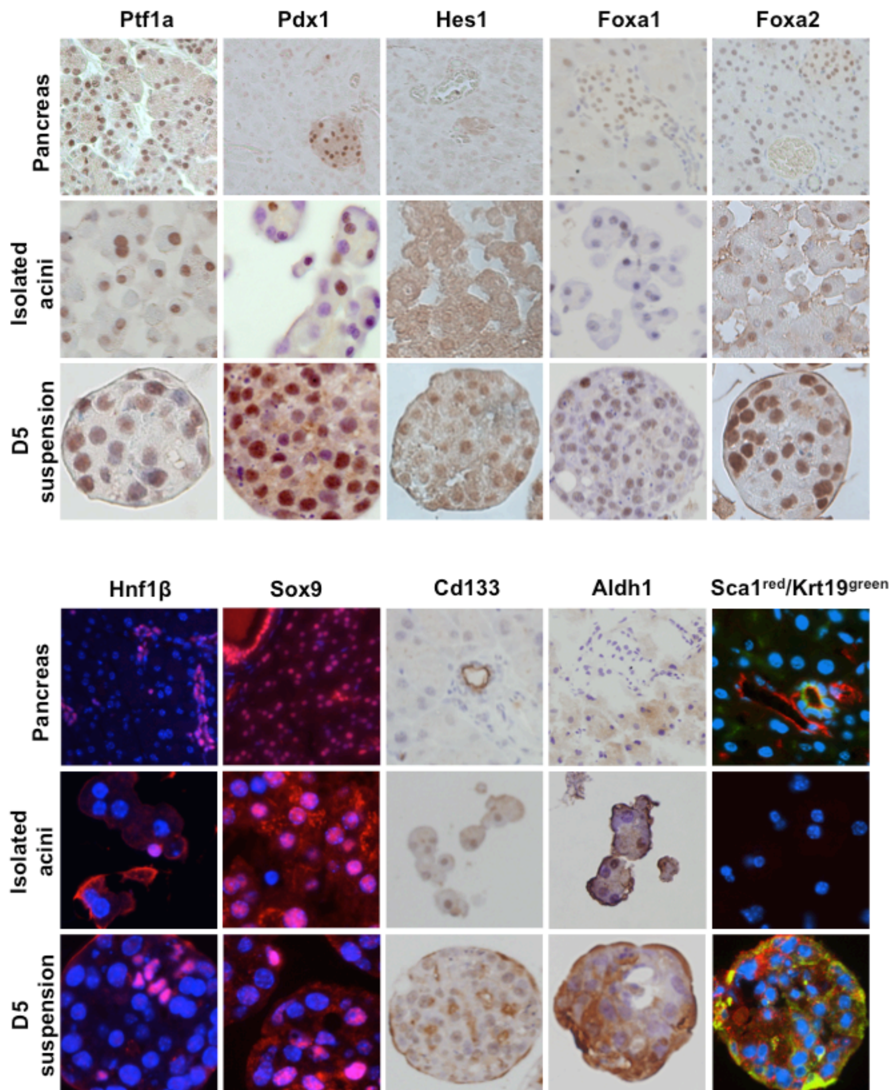

A
X-gal
Krt19
X-gal / Krt19
*


B
Isolated Acini
D5 suspension
Monolayer
Phase contrast
X-gal
Krt19

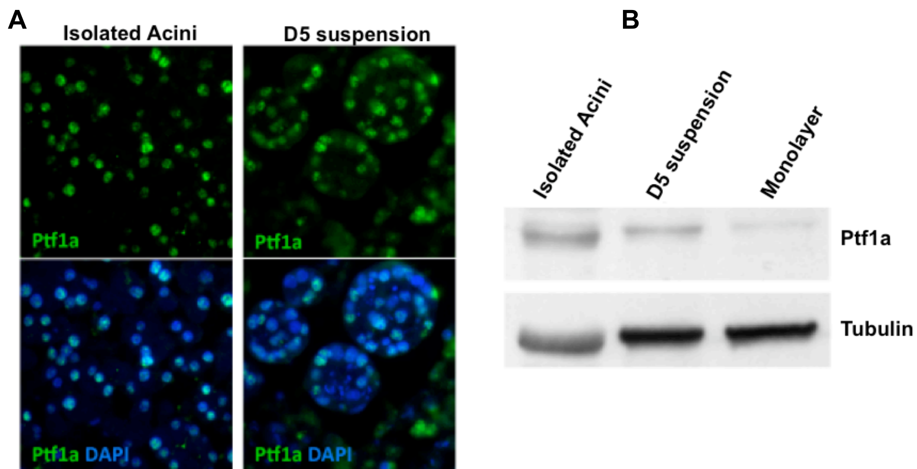
Supplementary Figure 2. Lineage tracing analysis of suspension and monolayer cultures. A. X-gal staining to detect β-galactosidase and Krt19 immunofluorescence were performed on total pancreas of $Ela^{CreERT2}$; R26R-LSL-LacZ mice after tamoxifen treatment. X-gal staining was absent from Krt19 positive ductal cells, but could not be excluded in those Krt19+ centroacinar (white asterisk) cells that are surrounded by X-gal+ acinar cells. **B.** Acinar fractions isolated from from $Ela^{CreERT2}$; R26R-LSL-LacZ mice treated with tamoxifen were cultured either in suspension or adherent to plastic. A double labeling was performed using X-gal staining to detect β-galactosidase and Krt19 immunofluorescence. The arrow indicates a small duct that is present in the isolated acinar sample, which is LacZ negative. In the suspension and adherent cultures, the Krt19 expressing cells stain positive for X-gal showing their acinar origin.



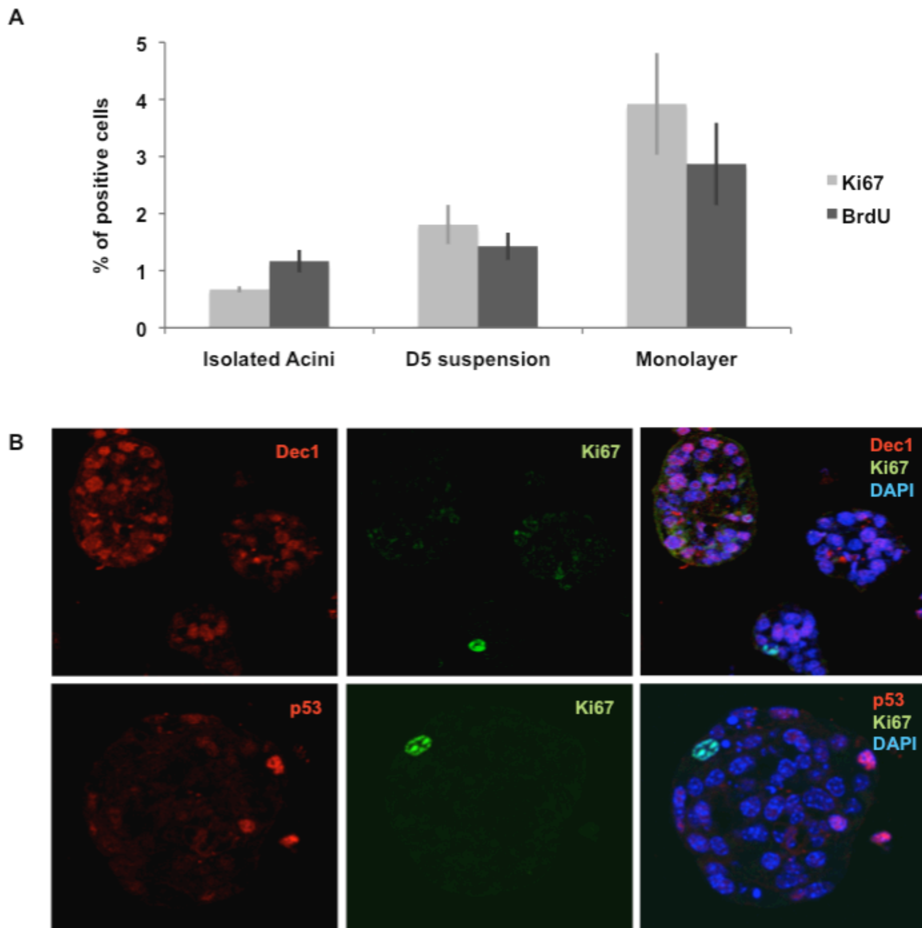
Supplementary Figure 3. Specificity analysis of antibodies detecting Rbpj and Rbpjl. HEK 293 cells were transfected with an empty vector, or with cDNAs coding for either Rbpj or Rbpjl; cell lysates were probed with antibodies detecting either protein using western blotting.



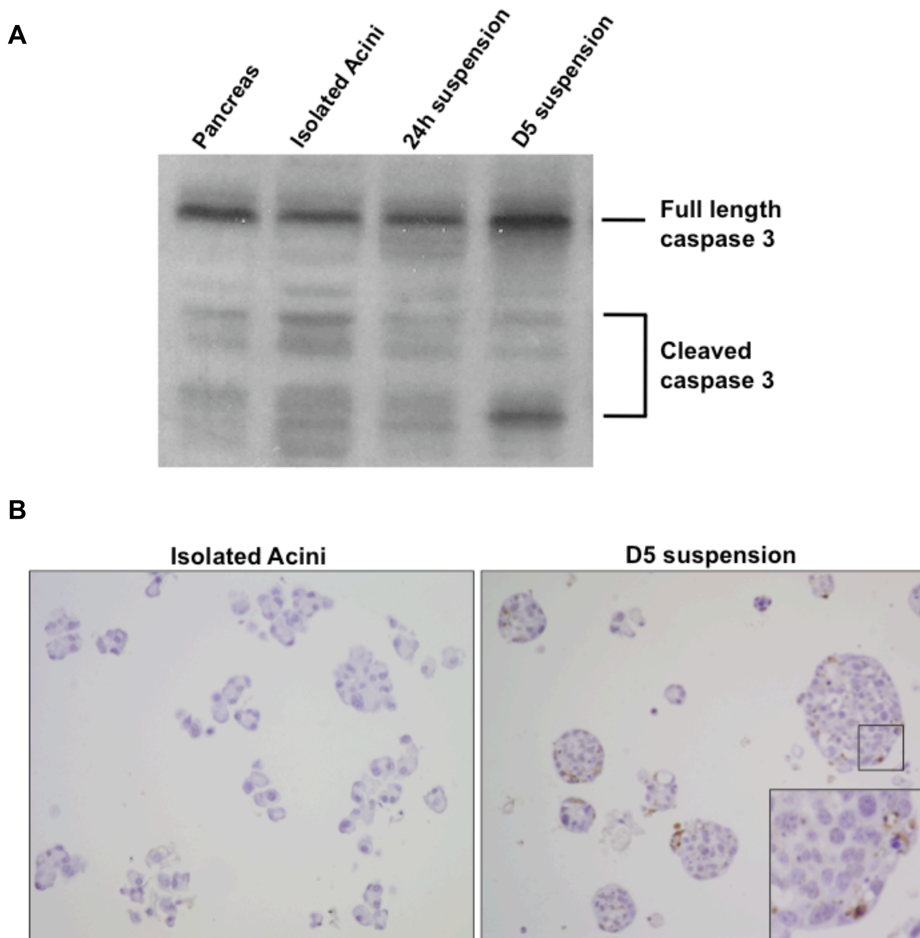
Supplementary Figure 4. Expression of markers of pancreatic progenitors and adult stem cells. Immunohistochemical and immunofluorescence analysis in normal pancreas tissue, isolated acini, and D5 suspension cultures. Nuclei were counterstained with hematoxylin or DAPI, except for Hes1.



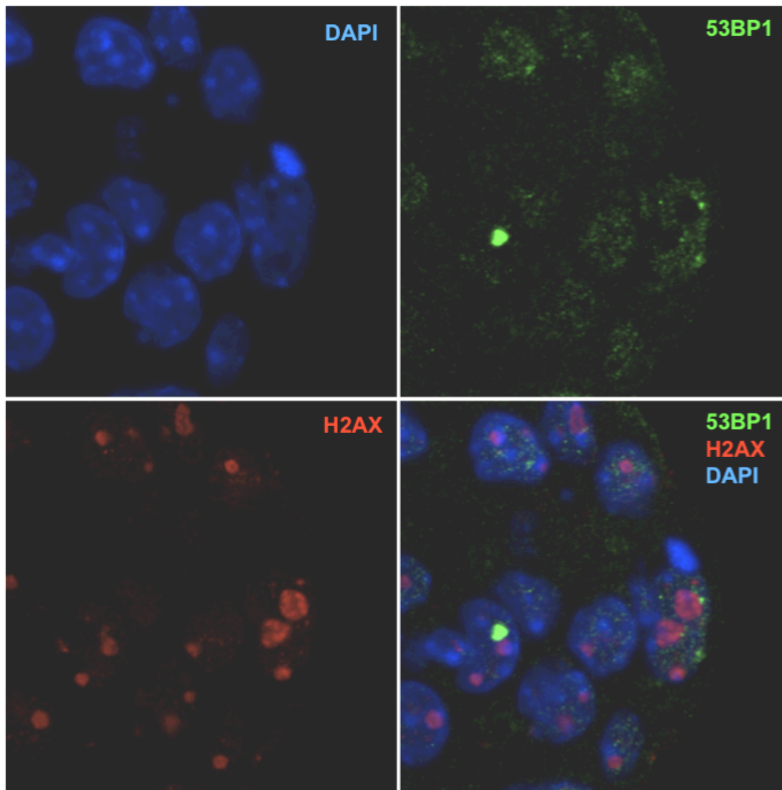
Supplementary Figure 5. Ptf1a expression in isolated acini, D5 suspension and monolayer cultures. A. Immunofluorescence for Ptf1a in isolated acini and D5 suspension cultures. **B.** Western blot for Ptf1a in isolated acini, D5 suspension and monolayer cultures. The decrease in expression of Ptf1a in D5 suspension cultures compared to isolated acini can be evidenced in the western blot analysis. Ptf1a is further decreased in monolayer cultures.



Supplementary Figure 6. Proliferation analysis in acinar cultures. A. Quantification of proliferative cells by Ki67 immunohistochemistry and 24h-BrdU uptake in isolated acini, D5 suspension and monolayer cultures. Results are represented as Mean±SEM (n=3-6). **B.** Double immunofluorescence of the proliferation marker Ki67 and the senescence markers Dec1 and p53 in D5 suspension cultures. Nuclei were counterstained with DAPI.

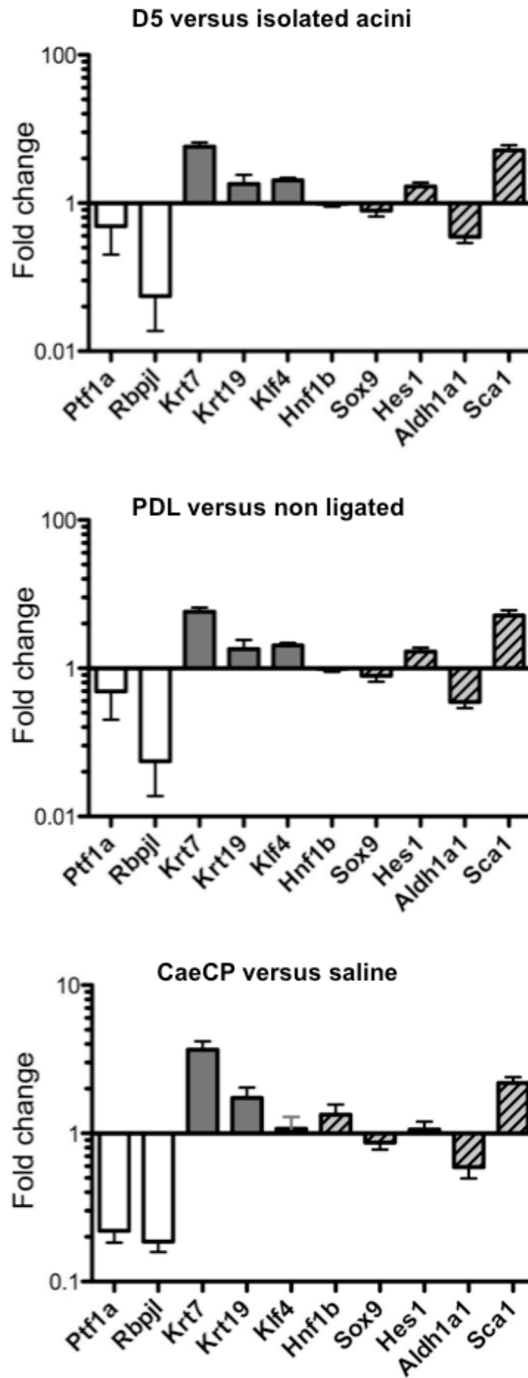


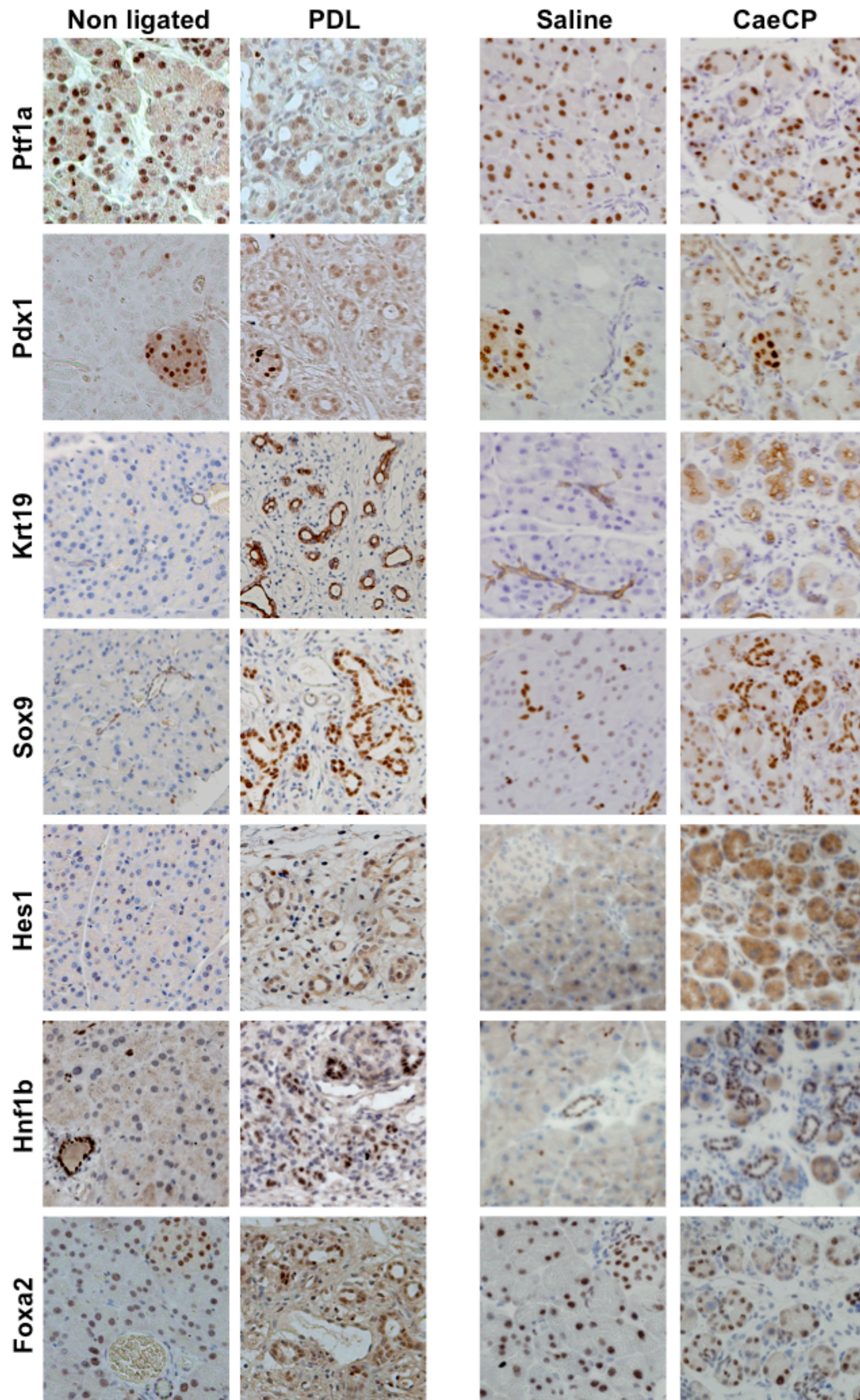
Supplementary Figure 7. Apoptosis in acinar cultures. A. Western blot analysis of the apoptosis marker caspase 3 in total pancreas, isolated acini, 24h and D5 suspension cultures. **B.** Immunohistochemistry of activated caspase 3, a marker of apoptosis, in isolated acini and D5 suspension cultures. Nuclei were counterstained with hematoxylin.



Supplementary Figure 8. DNA damage response in D5 suspension cultures. Double immunofluorescence of the DNA damage markers 53BP1 and phospho-γ-H2AX in D5 suspension cultures. Nuclei were counterstained with DAPI.

A




B
Non ligated
PDL
Saline
CaeCP
Ptf1a
Pdx1
Krt19
Sox9
Hes1
Hnf1b
Foxa2

Supplementary Figure 9. Experimental chronic pancreatitis tissues display an expression profile similar to the acini suspension cultures. A. qRT-PCR analysis of transcript levels in acinar cultures, pancreatic duct ligation (PDL), and caerulein-induced chronic pancreatitis (CaeCP). Transcript levels were compared to isolated acini, non-ligated pancreas, and saline-treated mice. Results normalized to Hprt. **B.** Immunohistochemical analysis of pancreatic progenitor cell markers in PDL compared with non-ligated pancreas and in CaeCP compared with saline-treated mice. Nuclei were counterstained with hematoxylin.

I.1.3. Supplementary Tables

	Gene Symbol	mRNA Accession	D5 / Acini	FDR indep	Description
Enzymes and other secretory proteins	Pla2g1b	NM_011107	0.09	0.0006	Phospholipase A2, group IB, pancreas
	Cel	NM_009885	0.23	0.0012	Carboxyl ester lipase
	Try4	NM_011646	0.21	0.0013	Trypsin 4
	Cela1	NM_033612	0.29	0.0013	Chymotrypsin-like elastase family, member 1
	Ctrl	NM_023182	0.32	0.0016	Chymotrypsin-like
	Cela3b	NM_026419	0.39	0.0022	Chymotrypsin-like elastase family, member 3B
	Klk1	NM_010639	0.29	0.0026	Kallikrein 1
	Pnliprp2	NM_011128	0.48	0.0034	Pancreatic lipase-related protein 2
	Try10	NM_001038996	0.35	0.0035	Trypsin 10
	Rnase1	NM_011271	0.49	0.0041	Ribonuclease, RNase A family, 1 (pancreatic)
	Klk1b8	NM_008457	0.29	0.0052	Kallikrein 1-related peptidase b8
	Cela2a	NM_007919	0.52	0.0052	Chymotrypsin-like elastase family, member 2A
	Reg3d	NM_013893	0.42	0.0057	Regenerating islet-derived 3 delta
	Prss3	NM_011645	0.36	0.0065	Protease, serine, 3
	Trypsinogen 15	NM_001103153	0.45	0.0075	Predicted gene 10334
	Prss1	NM_053243	0.51	0.0082	Protease, serine, 1 (trypsin 1)
	Try10l	NM_001003664	0.50	0.0138	Predicted gene 5409 (Gm5409)
	Cpb1	NM_029706	0.62	0.0203	Carboxypeptidase B1 (tissue)
	Pnliprp1	NM_018874	0.67	0.0225	Pancreatic lipase related protein 1
	Trypsinogen7	NM_023333	0.76	0.0373	RIKEN cDNA 2210010C04 gene (2210010C04Rik)
Mitochondrial metabolism	Gcat	NM_013847	0.21	0.0010	Glycine C-acetyltransferase (2-amino-3-ketobutyrate-coenzyme A ligase)
	Gatm	NM_025961	0.18	0.0012	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)
	Gls2	NM_001033264	0.26	0.0037	Glutaminase 2 (liver, mitochondrial)

Secretory protein modification, packaging, transport and exocytosis	Tmed11	NM_026109	0.04	0.0006	Transmembrane emp24 protein transport domain containing
	Zg16	NM_026918	0.14	0.0009	Zymogen granule protein 16
	Cuzd1	NM_008411	0.32	0.0017	CUB and zona pellucida-like domains 1
	Sycn	NM_026716	0.42	0.0026	Syncollin
	Derl3	NM_024440	0.42	0.0028	Der1-like domain family, member 3
	Dmbt1	NM_007769	0.46	0.0038	Deleted in malignant brain tumors 1
	Lfng	NM_008494	0.48	0.0042	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase
Miscelaneous	Slc38a5	NM_172479	0.03	0.0006	Solute carrier family 38, member 5
	Vtn	NM_011707	0.19	0.0008	Vitronectin
	Slc39a5	NM_028051	0.22	0.0012	Solute carrier family 39 (metal ion transporter), member 5
	Gal	NM_010253	0.10	0.0015	Galanin
	Tff2	NM_009363	0.29	0.0023	Trefoil factor 2 (spasmolytic protein 1)
	Slc6a9	NM_008135	0.39	0.0024	Solute carrier family 6 (neurotransmitter transporter, glycine), member 9
	Serinc3	NM_012032	0.64	0.0112	Serine incorporator 3
	Spink3	NM_009258	0.67	0.0181	Serine peptidase inhibitor, Kazal type 3
	Zp3	NM_011776	0.75	0.0369	Zona pellucida glycoprotein3
Acinar transcription factors	Rbpjl	NM_009036	0.21	0.0009	Recombination signal binding protein for immunoglobulin kappa J region-like
	Bhlha15 / Mist1	NM_010800	0.23	0.0011	Basic helix-loop-helix family, member a15

Supplementary Table 1. Expression of markers of the adult acinar differentiation programme in D5 suspension cultures compared to isolated acini. This gene set was selected from the transcripts significantly down-regulated in the pancreas of Rbpjl null mice (Masui et al. 2010). Affymetrix expression array data analysed using a limma t-test (Pomelo II).

Gene Symbol	mRNA Accession	D5 / Ducts	FDR indep	mRna - Description
Atp1b1	NM_009721	0.17	0.0000	ATPase, Na+/K+ transporting, beta 1 polypeptide
Onecut1	NM_008262	0.35	0.0000	One cut domain, family member 1
Hnf1b	NM_009330	0.36	0.0001	HNF1 homeobox B
Car2	NM_009801	0.55	0.0032	Carbonic anhydrase 2
Cftr	NM_021050	0.59	0.0175	Cystic fibrosis transmembrane conductance regulator homolog
Anxa2	NM_007585	0.93	0.6150	Annexin A2
S100a10	NM_009112	1.25	0.1712	S100 calcium binding protein A10
Krt7	NM_033073	2.25	0.0010	Keratin 7
Krt19	NM_008471	2.43	0.0000	Keratin 19

Supplementary Table 2. Expression of duct-related markers in D5 suspension cultures compared to isolated ducts. Affymetrix expression array data analysed using a limma t-test (Pomelo II).

Gene	Forward primer	Reverse primer
Hprt	GGCCAGACTTTGTTGGATTTG	TGCGCTCATCTTAGGCTTTGT
Ptf1a	ACAAGCCGCTAATGTGCGAGA	TTGGAGAGGCGCTTTTCGT
Rbpjl	ATGCCAAGGTGGCTGAGAAAT	CTTGGTCTTGCATTGGCTTCA
Mist1	CCTTCAACTCTCCAGGGAAA	GCCACCACACATGCAATT
Rbpj	GTGTTCCTCAGCAAGCGGATA	TGCCACCTTCGTTCCTGA
Pcaf	TGGAGAAGAAGCCGCCATTT	TCGTTGTCTGCCTCTCTTTCG
Cpa1	TACACCCACAAAACGAATCGC	GCCACGGTAAGTTTCTGAGCA
Ctrb1	GCAAGACCAAATACAATGCCC	TGCGCAGATCATCACATCG
Amy2	TGGCGTCAAATCAGGAACATG	AAAGTGGCTGACAAAGCCCAG
Ela1	CGTGGTTGCAGGCTATGACAT	TTGTTAGCCAGGATGGTTCCC
Krt7	CACGAACAAGGTGGAGTTGGA	TGTCTGAGATCTGCGACTGCA
Krt19	CCTCCCGCGATTACAACCACT	GGCGAGCATTGTCAATCTGT
Klf4	CCAGACCAGATGCAGTCACAA	ACGACCTTCTTCCCCTCTTTG
Hes1	TCCAAGCTAGAGAAGGCAGACA	CGTTCATGCACTCGCTGAA
Foxa1	GAACAGCTACTACGCGGACA	TGGTCATGTAGGTGTTCATGG
Foxa2	CTGGGAGCCGTGAAGATGGA	CCAGCGCCCACATAGGATGA
Hnf1b	TACGACCGGCAAAAGAATCC	TGCGAACCAGTTGTAGACACG
Sox9	CGTGCAGCACAAGAAAGACCA	GCAGCGCCTTGAAGATAGCAT
c-met	CATCCCAATGTTCTCTCACT	TTGAAGGCCAAATCCTATAA
Cd133	CTAGAAGAGGCTGTGTGTCC	TTTGCAATCCCTGTAGACTT
Aldh1a1	TTCTTAACCCTGCAACTGAG	AGTATGCATTGGCAAAGACT
c-kit	TCATCGAGTGTGATGGGAAA	GGTGACTTGTTTCAGGCACA
Sca1	TCCTGTTTGCTGATTCTTCT	TTCAATATTAGGAGGGCAGA

Supplementary Table 3. List of primers used for RT-qPCR.

Gene	Forward primer	Reverse primer
R28S	CTGGGACATAGTGGGTGCTT	GAGCCTAGAGATGGGCTGTG
promoter Cpa1	CCATGGTCAAGGGTGAAAGC	TCTGGGGCCTTTTTAAACAC
promoter Rpbjl	TGCTGGGTCTGGCTTCTACT	CCGATCCTCACACTGGATTT
promoter Ptf1a (prox. enhancer)	TGTGTTATGATTCCCACGGACT	TACCTGAGCCCTTGACTGGTAA

Supplementary Table 4. List of primers used for ChIP.

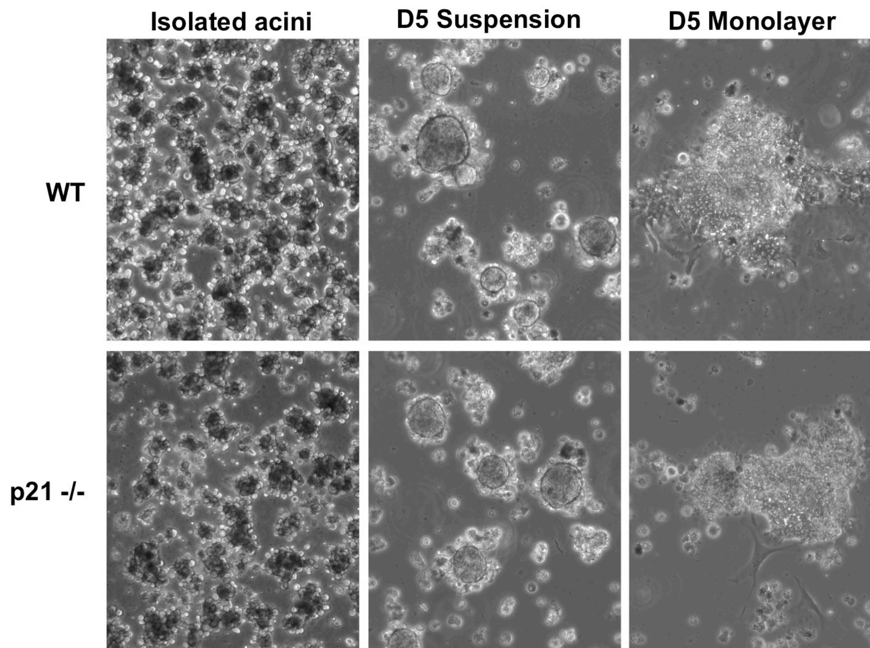
II. Research Publication 2

p53-dependent regulation of growth, epithelial-mesenchymal transition and stemness in normal pancreatic epithelial cells

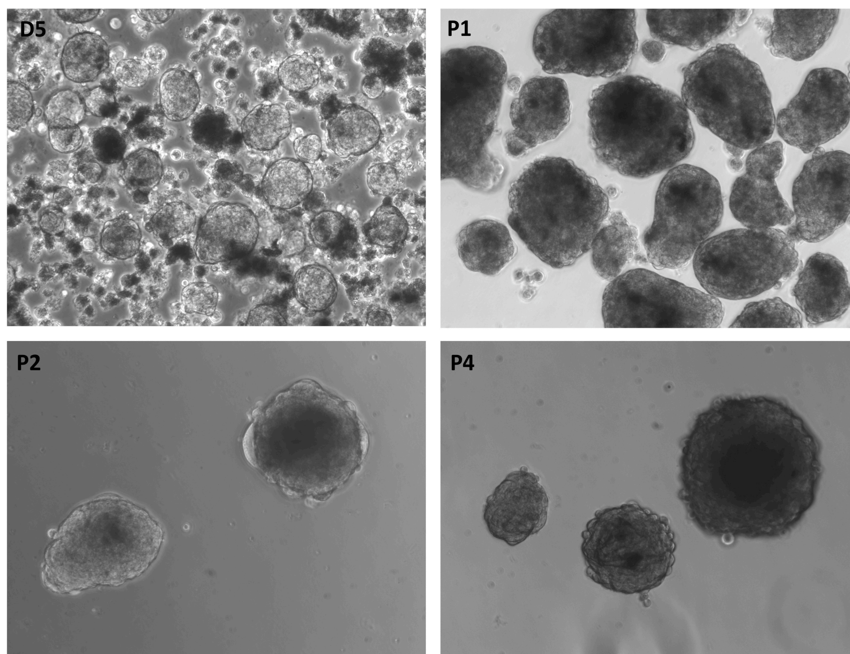
Pinho AV, Rooman I, Real FX. p53-dependent regulation of growth, epithelial-mesenchymal transition, and stemness in normal pancreatic epithelial cells. Cell Cycle, 2011; 10(8):1312-1321.

Pinho AV, Rooman I, Real FX. p53-dependent regulation of growth, epithelial-mesenchymal transition and stemness in normal pancreatic epithelial cells. Cell Cycle. 2011; 10(8): 1312-21.

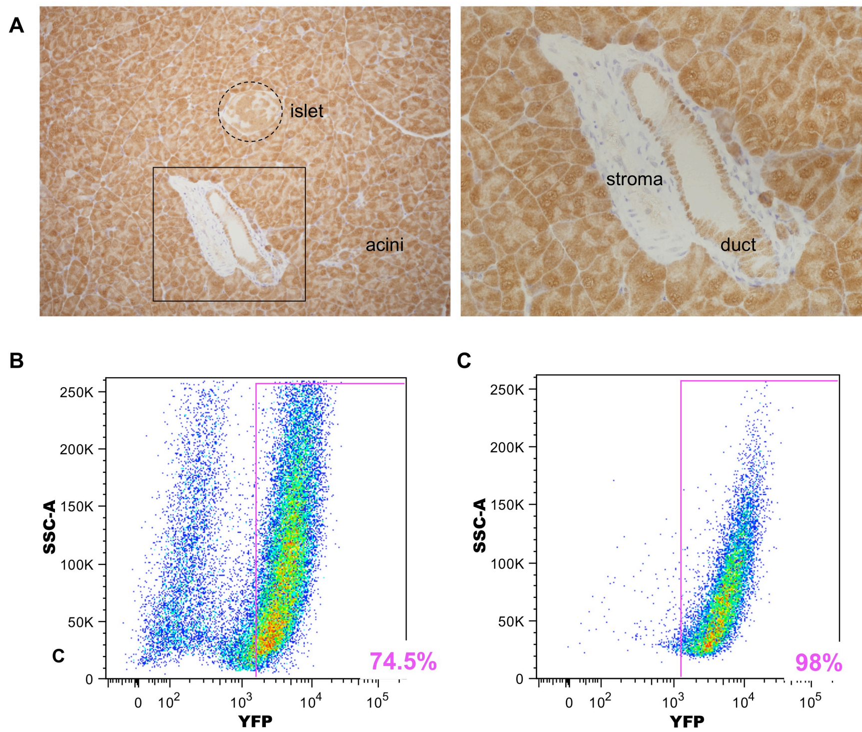
II.1. Supplementary Material



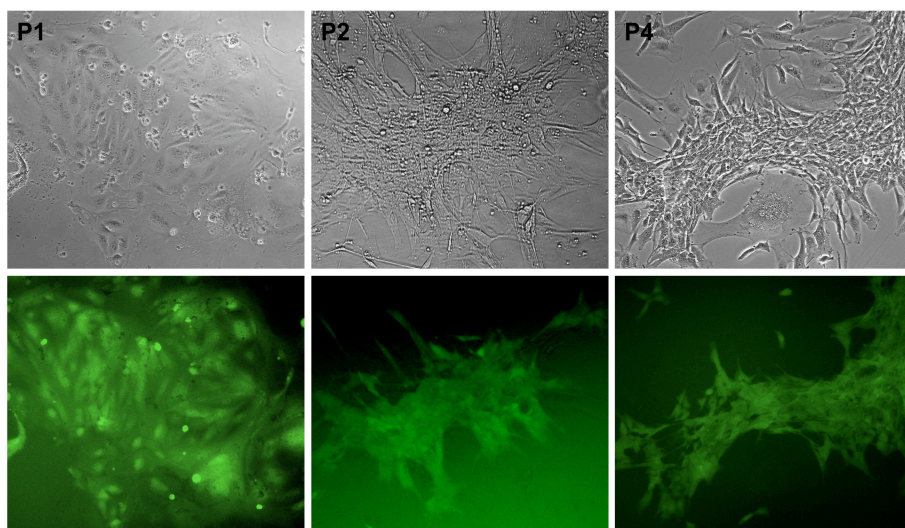
Supplementary Figure 1. Acinar cell cultures from p21$^{-/-}$ mice do not have enhanced proliferative capacity. Acinar fractions from p21$^{-/-}$ mice display primary sphere formation capacity and growth as monolayer similar to wild type control cells.



Supplementary Figure 2. p53$^{-/-}$ cells can be expanded in suspension culture. Morphological appearance of primary cultures after 5 days of culture (D5) and secondary spheres formed after consecutive passages (P1, P2 and P4). All images were taken at 100x magnification.



Supplementary Figure 3. Lineage tracing of pancreatic epithelial cells from Ptf1a^{Cre};R26R-LSL-EYFP;p53$^{-/-}$ mice. A. Immunohistochemical analysis of GFP in pancreas from Ptf1a^{Cre};R26R-LSL-EYFP;p53$^{-/-}$ mice shows that EYFP is expressed exclusively in pancreatic epithelial cells (acini, islets and ducts) and not in the stroma. **B.** Flow cytometry analysis of primary cultures after dissociation. 74.5% of cells with high expression of EYFP were sorted and seeded. **C.** Flow cytometry analysis of P12 cultures. 98% of cells with high expression of EYFP were sorted and seeded for immunofluorescence analysis.



Supplementary Figure 4. Monolayer cultures of pancreatic cells from Ptf1a^{Cre};R26R-LSL-EYFP;p53$^{-/-}$ mice. Phase contrast microscopy and EYFP expression of Ptf1a^{Cre};R26R-LSL-EYFP;p53$^{-/-}$ cells, sorted at P1 and analyzed at subsequent passages (P1, P2 and P4). All images were taken at 100x magnification. This cells line was used for the mRNA analysis shown in Figure 4A.

II.2. Appendix

Caerulein-induced acute pancreatitis in p53$^{-/-}$ mice

II.2.1. Appendix Materials and Methods

Caerulein-induced acute pancreatitis. Acute pancreatitis was induced in 8-week old C57BL/6JOlaHsd mice (Harlan Europe), referred to as wild type and in *Trp53* null [304], referred to as p53$^{-/-}$. Caerulein treatment was performed through the administration of 8 hourly intraperitoneal injections of 2μg of caerulein (Sigma) during 2 consecutive days as previously described [123]. Animals were sacrificed and blood and pancreatic tissue were recovered at 0h (non-treated animals), 48h and 8 days (8d) after the first caerulein administration. All animal procedures were performed in accordance with institutional ethical committees and national guidelines and regulations.

Histological grading of acute pancreatitis lesions. Formalin-fixed paraffin-embedded (FFPE) pancreatic tissue was sectioned and stained with hematoxylin and eosin. Sections were graded blinded by an expert clinical pathologist. The extent and severity of edema, inflammatory cell infiltrate, acinar vacuolization and formation of tubular complexes necrosis were graded from 0–3. The histological score is available in Appendix Table 1.

Analysis of proliferation. Ki67 immunohistochemistry was performed on FFPE sections of pancreas as described previously in Research Publication 2 [382]. Rabbit anti-Ki67 (NCL-Ki67p, Leica Biosystems) antibody was used at a concentration 1:1000. Photographs were taken from random areas of each sample, having in consideration that the whole field was occupied by pancreatic tissue. Ki67^{+} cells were counted using ImageJ software. Results

are shown as the sum of Ki67^{+} positive cells in the area corresponding to 4 fields.

RT-qPCR. Total RNA was isolated, treated with DNase and reverse-transcribed as previously described [382]. Real time PCR was performed using 30ng of RNA-equivalent in the presence of Power SYBR Green PCR Master Mix (Invitrogen) and results were analysed as described in Research Publication 2 [382]. The expression levels were normalized to individual Hprt expression. List of primers used for RT-qPCR in Research Publication 2 and in Annex Results is shown in Appendix Table 2.

Assay of serum pancreatic enzymes. Total blood was collected from the facial vein previously to animal sacrifice. Blood was left to clot for 2h at room temperature, after which was centrifuged at 2000g for 20 min and the serum was collected. Amylase and lipase serum levels were measured at the Service of Clinical Chemistry of UZ Brussel, Brussels, Belgium.

Statistical analyses. Data was analyzed using Mann-Whitney test and statistical significance was accepted at a confidence interval <0.05 (InStat GraphPad software). Results are shown as Mean±SEM and n=4/5.

Condition	**Score**	**Indication**
Edema	0	Absent
	1	Focal between lobules
	2	Diffuse between lobules
	3	Intralobular with acini disruption
Inflammation	0	Absent
	1	Focal periductal
	2	In the parenchyma (in <50% of lobules)
	3	In the parenchyma (in >50% of lobules)
Acini vacuolization	0	Absent
	1	In <10% of acinar cells
	2	In 10-50% of acinar cells
	3	In >50% of acinar cells
Tubular complexes	0	Absent
	1	Periductal
	2	Focal (in <50% of the lobules)
	3	Diffuse (in >50% of the lobules)

Appendix Table 1. Histological score for evaluation of acute pancreatitis.

Gene	Forward primer	Reverse primer
Hprt	GGCCAGACTTTGTTGGATTTG	TGCGCTCATCTTAGGCTTTGT
Ptf1a	ACAAGCCGCTAATGTGCGAGA	TTGGAGAGGCGCTTTTCGT
Cpa1	TACACCCACAAAACGAATCGC	GCCACGGTAAGTTTCTGAGCA
Amy2	TGGCGTCAAATCAGGAACATG	AAAGTGGCTGACAAAGCCCAG
Krt7	CACGAACAAGGTGGAGTTGGA	TGTCTGAGATCTGCGACTGCA
Krt19	CCTCCCGCGATTACAACCACT	GGCGAGCATTGTCAATCTGT
Pdx1	AAATCCACCAAAGCTCACGC	CGGTCAAGTTCAACATCACTGC
Foxa2	CTGGGAGCCGTGAAGATGGA	CCAGCGCCCACATAGGATGA
Hnf1b	TACGACCGGCAAAAGAATCC	TGCGAACCAGTTGTAGACACG
Sox9	CGTGCAGCACAAGAAAGACCA	GCAGCGCCTTGAAGATAGCAT
Cd133	CTAGAAGAGGCTGTGTGTCC	TTTGCAATCCCTGTAGACTT
Klf4	CCAGACCAGATGCAGTCACAA	ACGACCTTCTTCCCCTCTTTG
c-myc	CCTAGTGCTGCATGAGGAGAC	CCTCATCTTCTTGCTCTTCTTCA
Gata6	GAGCCTGTGTGCAATGCTT	GGTTTTCGTTTCCTGGTTTG
Sox17	GTTGGCACAGCAGAACCCAG	TCATGCGCTTCACCTGCTTG
Hhex	CGGTGAACGACTACACGCAC	CTCTCGGGTGGGGAGAGGTA
Bmi1	AGTAAATAAAGAGAAGCCTAAGGAAGAG	TTCTCAAGTGCATCACAGTCATT
Abcg2	TTCCTAGGATGAACTCCAGA	TCTTTCCTTGCTGCTAAGAC
Twist	CGGGTCATGGCTAACGTG	CAGCTTGCCATCTTGGAGTC
Snai1	CCGGAAGCCCAACTATAGCGA	ACAGCGAGGTCAGCTCTACG
Snai2	GCTTTCTCCAGACCCTGGCT	TGCAGATGTGCCCTCAGGTT
Zeb1	GAGCCGCCAGTGAAGGTGATC	GTGAGGCCTCTTACCTGTGTGCT
Zeb2	CAGTTCTAACCTGCTCGGCAGG	AGCGGATCAGATGGCAGTTCG
E-caderin	ACGGAGGAGAACGGTGGTCA	TGAGGGTGGGAGCCACATCA
Vimentin	CGGAAAGTGGAATCCTTGCA	CCACATCGATCTGGACATGCT
N-cadherin	TGGGTCATCCCGCCAATCAA	AACCGGGCTATCAGCTCTCG

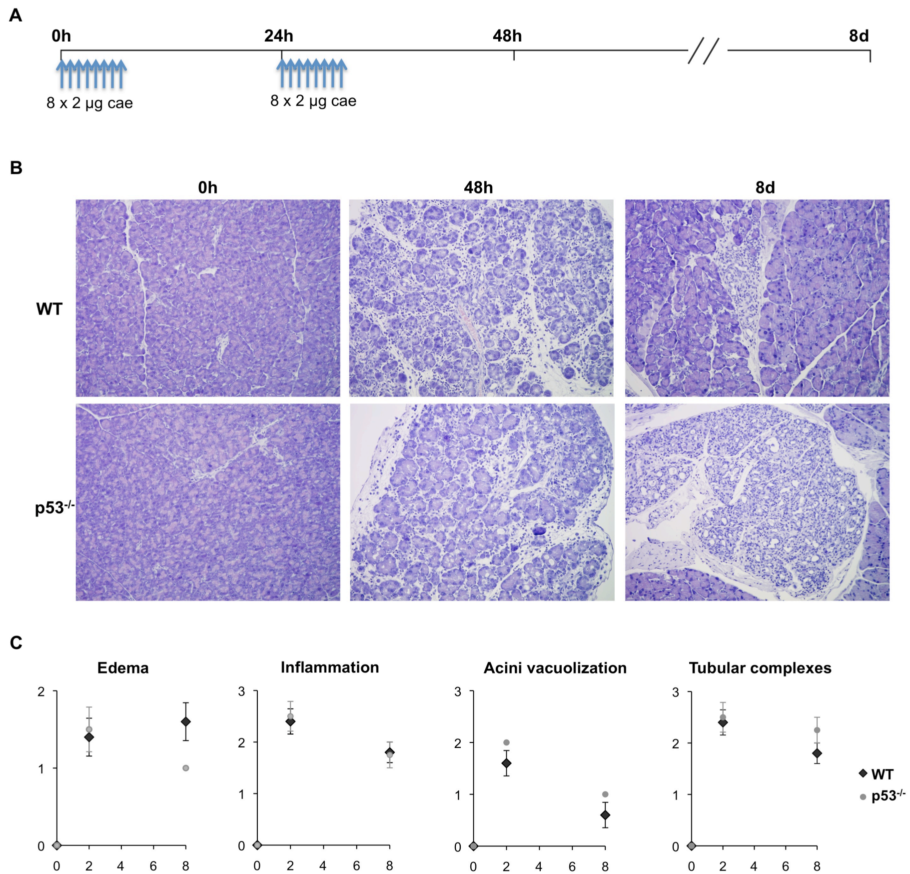
Appendix Table 2. List of primers used for RT-qPCR in Research Publication 2 and Appendix Results.

II.2.2. Appendix Results

To analyze to effect of the absence of p53 *in vivo* and after pancreatic acinar cell injury, acute pancreatitis was induced in wild type (WT) and $p53^{-/-}$ animals by administration of caerulein, using a 2-day protocol previously shown to induce the dedifferentiation of acinar cells [123] (Appendix Figure 1.A). Animals were sacrificed at 0h (non-treated), 48h and 8 days (8d) after the initiation of treatment.

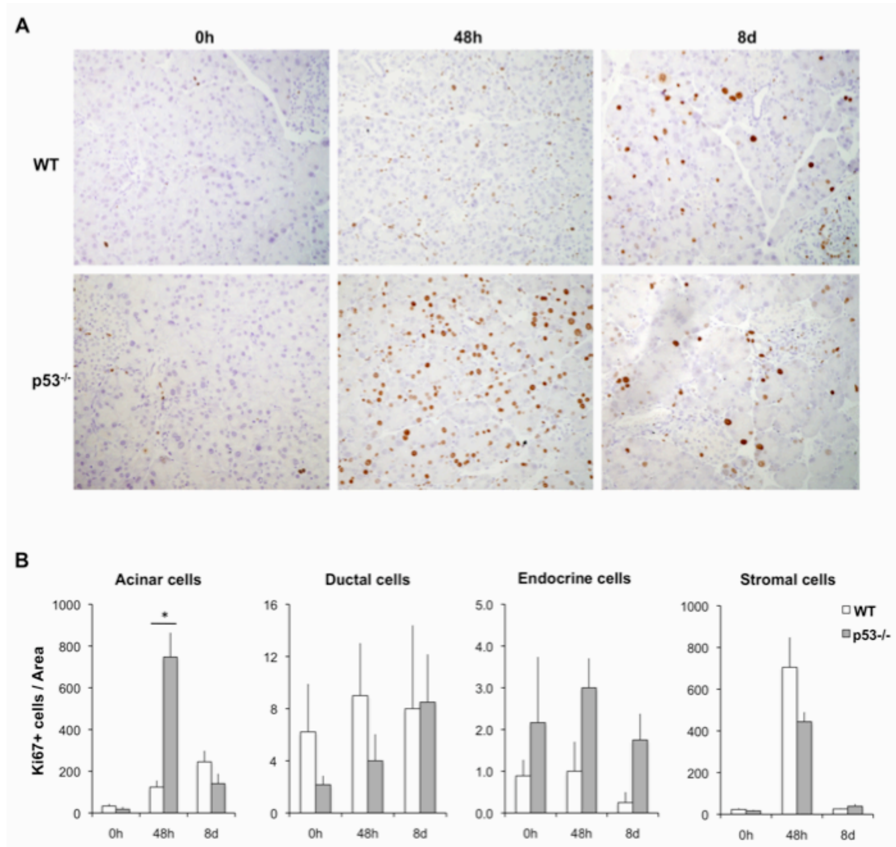
To evaluate the level of histological injury before and after caerulein treatment pancreatic tissue sections of wild type and $p53^{-/-}$ animals were graded by a clinical pathologist (Appendix Figure 1.B and C) according to the histological score shown on Appendix Table 1. Pancreata from $p53^{-/-}$ non-treated animals (0h) were similar to non-treated wild type animals, presenting no evident histological alterations. 48h after initiation of treatment both groups of animals presented severe histological alterations commonly associated with acute pancreatitis, including edema, presence of inflammatory cell infiltrates, acini vacuolization and formation of tubular complexes. The type of lesions present in $p53^{-/-}$ was similar to that observed in control animals. Nevertheless, $p53^{-/-}$ pancreata showed a slight increase in acinar vacuolization. Eight days after caerulein treatment both groups of animals had partially recovered from the damage. $p53^{-/-}$ animals showed reduced edema but a small increase in acinar cell vacuolization and in the presence of tubular complexes, which was not statistically significant.

To evaluate whether lack of p53 had an effect on pancreatic cell proliferation *in vivo* after caerulein-induced acute pancreatitis, Ki67 immunohistochemistry was performed on pancreas sections and the number of $Ki67^{+}$ proliferative nuclei was counted (Appendix Figure 2).



Appendix Figure 1. Caerulein-induced acute pancreatitis in p53$^{-/-}$ mice: analysis of pancreas histological alterations. A. Caerulein was administered in 8 hourly 2µg doses during 2 consecutive days and animals were analyzed 0h, 48h and 8 days (8d) after initiation of treatment. **B.** Hematoxylin and eosin staining of pancreata from wild type (WT) and p53$^{-/-}$ mice at 0h, 48h and 8d after initiation of caerulein treatment. **C.** Histological score for the presence of edema, inflammation, acini vacuolization and tubular complexes in pancreata from wild type (WT) and p53$^{-/-}$ mice at 0h, 48h and 8d after initiation of caerulein treatment. For all parameters a score ranging from 0-3 was used (see Appendix Table 1). Results are shown as Mean± SEM; n = 4 / 5.

Non-treated p53$^{-/-}$ animals presented no statistically significant differences in the number of Ki67^{+} cells/area in either acinar (17.8±11.3), ductal (2.2±0.7), endocrine (2.2±1.6) or stromal (16.5±4.5) cells, when compared with wild type animals (33.3±10.5; 6.2±3.7; 0.9±0.4 and 23.1±6.3, respectively).

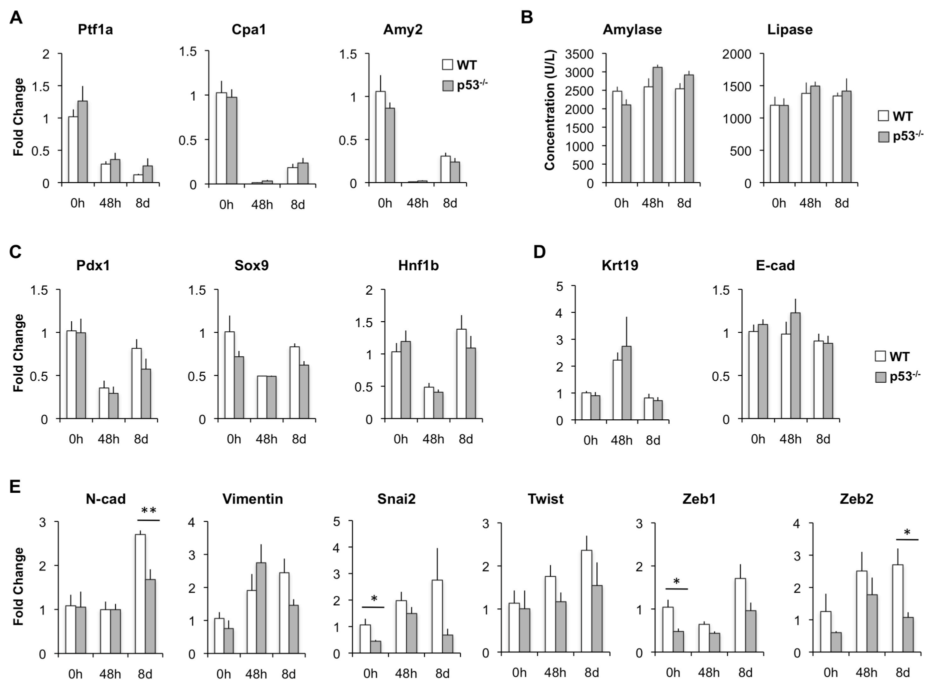


Appendix Figure 2. Caerulein-induced acute pancreatitis in p53-/- mice: analysis of proliferation. A. Ki67 immunohistochemistry in pancreata from wild type (WT) and p53$^{-/-}$ mice at 0h, 48h and 8d after initiation of caerulein treatment. Nuclei are counterstained with hematoxylin. Magnification = 200X**. B.** Quantification of Ki67+ acinar, ductal, endocrine and stromal cells per area (corresponding to 4 microscope fields). Results are shown as Mean±SEM; n=4; *p<0.05.

48h after the initiation of caerulein treatment, p53$^{-/-}$ animals presented a very pronounced increase in acinar cell proliferation with 747.3±116.7 Ki67^{+} cells/area unit, compared with 124±31.5 Ki67^{+} cells/area unit in wild type animals ($p<0.05$). At the same time point, no statistically significant effect was seen regarding the proliferation of ductal (9±4 in WT vs 4±2 in p53$^{-/-}$), endocrine (1±0.7 in WT vs 3±0.7 in p53$^{-/-}$) or stromal (704.8±143.8 in WT vs 444.3±45.6 in p53$^{-/-}$) cells. This shows that there is a p53-dependent effect of caerulein treatment on proliferation that is specific for pancreatic acinar cells. 8 days after initiation of treatment, the number of proliferative acinar cells in p53$^{-/-}$ animals decreased to 140.8±47.8, presenting no statistically significant differences from wild type controls (245±53.3). Again, there was no statistically significant alteration in the number of Ki67^{+} cells of ductal, endocrine or stromal cells in p53$^{-/-}$ animals (8.5±3.7, 1.8±0.6 and 38.8±10.3 respectively) compared with controls (8±6.4, 0.3±0.3 and 26±2.9 respectively).

To determine wether the induction of acute pancreatitis in p53$^{-/-}$ animals could lead to increased acinar cell dedifferentiation and/or to epithelial-mesenchymal transition *in vivo*, we analysed the level of expression of transcripts for acinar, pancreatic progenitor, epithelial and mesenchymal cell markers in total RNA from pancreas tissue, as well as the protein levels in serum of pancreatic enzymes (Appendix Figure 3). Regarding the mRNA expression of the acinar cells genes *Ptf1a*, *Cpa1* and *Amy2* (Appendix Figure 1.A) and the serum protein levels of the acinar enzymes amylase and lipase (Appendix Figure 1.B), no statistically significant differences were found between wild type and p53$^{-/-}$ animals either untreated, or 48h or 8d after caerulein treatment. Similarly, the levels of transcripts for the pancreatic progenitor genes *Pdx1*, *Sox9* and *Hnf1b* (Appendix Figure 1.C) and for the ductal cell marker *Krt19* and the epithelial marker E-cadherin (*E-cad*)

(Appendix Figure 1.D) presented no statistically significant differences between wild type and p53-null animals in all the time points analyzed. The transcript levels of the mesenchymal markers N-cadherin (*N-cad*) and Vimentin, and of the EMT regulators *Snai2*, *Twist*, *Zeb1* and *Zeb2* were also analysed and seem to indicate a tendency for being lower in p53$^{-/-}$ animals compared to wild type controls. Nevertheless, a statistically significant downregulation in the absence of p53 was only found for the genes *Snai2* (1.1±0.23-fold in WT vs 0.5±0.05-fold in p53$^{-/-}$; $p<0.05$) and *Zeb1* (1.0±0.17-fold in WT vs 0.5±0.07-fold in p53$^{-/-}$; $p<0.05$), in untreated animals; and for *N-cad* (2.7±0.09-fold in WT vs 1.7±0.23-fold in p53$^{-/-}$; $p<0.01$) and *Zeb2* (2.7±0.51-fold in WT vs 1.1±0.16-fold in p53$^{-/-}$; $p<0.05$), 8h after caerulein treatment. Altogether these results indicate that the induction of acute pancreatitis in p53-null animals did not cause increased acinar cell dedifferentiation or EMT.



Appendix Figure 3. Caerulein-induced acute pancreatitis in p53$^{-/-}$ mice: expression of acinar, pancreatic progenitor, epithelial and mesenchymal cell markers. RT-qPCR analysis of transcripts coding for acinar (**A**), pancreatic progenitor (**C**), epithelial (**D**) and mesenchymal (**E**) cell markers in wild type and p53$^{-/-}$ pancreatic tissue collected 0h, 48h and 8 days (8d) after initiation of caerulein treatment. **B**. Serum levels of the acinar enzymes amylase and lipase 0h, 48h and 8 days after initiation of caerulein treatment. Results are shown as Mean±SEM, n=4/5, *$p<0.05$, **$p<0.01$.

DISCUSSION

I. Adult pancreatic acinar cells dedifferentiate into pancreatic progenitor-like cells

Pancreatic acinar cells have been described to possess the ability to reprogram into other differentiated pancreatic cell types, such as ductal[85, 88, 91] and β-cells[92-94], and into non-pancreatic cells, such as hepatocytes[100, 101] and adipocytes[51]. These studies have thus unveiled the capacity of pancreatic acinar cells to undergo transdifferentation, a process that is defined by the conversion of one mature cell type into another differentiated cell type [383]. On the other hand, dedifferentiation involves a terminally differentiated cell reverting back to a less differentiated stage from within its own lineage [384]. In this study, we have used a combination of techniques such as lineage tracing, assessment of transcript and protein expression, analysis of promoter occupancy by transcription factors, and global transcriptomics to demonstrate that pancreatic acinar cells dedifferentiate - rather than transdifferentiate - into an embryonic pancreatic progenitor phenotype.

By performing lineage tracing with acinar cell fractions isolated from $Ela^{CreERT2}$;R26R-LSL-LacZ mice, we showed that the dedifferentiated cells forming the D5 suspension spheres originate from Elastase+ acinar cells. Upon culture, acinar cells very rapidly downregulated the expression of acinar-specific genes such as Mist1, Rbpjl and the digestive enzymes. Very interestingly, D5 suspension acinar cultures maintained the expression at low levels of Ptf1a, which is co-expressed together with Pdx1. This is a characteristic of multipotent pancreatic progenitor cells, since in the adult pancreas, Ptf1a expression is restricted to differentiated acinar cells [385], while Pdx1 is strongly expressed only in the endocrine compartment [26]. Furthermore, both Pdx1 and Ptf1a have been shown to be essential for pancreas formation [22, 23, 29], being activated early in development in the cells

that become fated to form the pancreas. The endoderm genes Foxa1 and Foxa2, which are known inducers of Pdx1, being required for pancreas formation [37], were also expressed in the D5 suspension cultures. In addition, D5 suspension cultures activated the expression of the Notch target Hes1, maintained the expression of Cpa1 at low levels, and expressed Hnf1b and Sox9, all these being genes previously shown to be expressed in multipotent pancreatic progenitor cells during embryonic development [44, 46, 47, 50, 55]. Markers that label adult populations of pancreatic progenitor cells, such as c-met, Cd133, Aldh1a, c-kit, and Sca-1 [6, 386, 387], were also expressed by D5 suspension cells. Klf4, which in the adult is present in ductal cells [388], and has been found to be related with the acquisition of stemness properties [336], was also strongly increased in D5 suspension cultures. Bmi1, a member of the polycomb-repressive complex 1 (PRC1) shown to be involved in the self-renewal of stem cells in several systems[389], including the pancreas [390], had also been previously shown to be upregulated in a similar model [391], further supporting the acquisition of stemness properties in these cells.

Nevertheless, relying only in marker expression analysis has limitations, namely the fact that we may be detecting mRNA or protein that is not being synthesized at the moment of analysis. Thus, we performed ChIP to assess the presence of PTF1 transcriptional complex components at the promoters of genes regulated by this complex. As expected, in isolated acini we found Ptf1a and Rbpjl, the components of the adult-type PTF1 (aPTF1) complex, characteristic of mature acinar cells. On the other hand, in D5 suspension cultures the promoters of Cpa1 and Ptf1a were occupied by Ptf1a and Rbpj, showing that Rbpj has replaced Rbpjl in the complex and therefore, these cells display a programme characteristic of the embryonic-type PTF1 (ePTF1) complex. Furthermore, global transcriptome analysis revealed that

D5 suspension cultures showed a downregulation of many of the genes that were found to be decreased in pancreata from mice lacking the expression of Rbpjl, which maintain the ePTF1 complex throughout development and consequently have an incomplete acinar cell differentiation [76].

Previously, other studies had reported the transdifferentiation of acinar primary cultures to ductal cells [82-85]. The fact that acinar-derived cells upregulate ductal keratins 7 and 19, which in the adult pancreas are markers of differentiated ducts, might lead to a misinterpretation of the phenotype. Nevertheless, these keratins are also expressed in embryonic pancreas during branching morphogenesis[392], being lost later during acinar and endocrine differentiation. The results obtained in the global transcriptome comparison of D5 acinar cultures with isolated ductal cells clearly show that primary acinar cultures do not display a ductal phenotype. In fact, more than 7000 genes were differentially expressed between D5 suspension cultures and isolated ductal cells and the majority of genes that were upregulated in normal ductal cells compared to acinar cells were not upregulated in D5 suspension cultures. Principal component analysis further supports the conclusion that D5 suspension cultures are phenotypically distinct from both acinar and ductal differentiated cells since these samples cluster neither with isolated acini nor with isolated ducts. A direct comparison between D5 suspension cultures and embryonic multipotent progenitor cells is still lacking and would be important to further characterize the acinar cell cultures.

Some of the previous studies were performed using different conditions as the cells were cultured as monolayers [82, 83] or embedded in a 3D matrix [85]. The suspension cultures used here allowed the formation of spheres, a method that has been shown to select for cells with stemness properties[393].

This technique is largely used to culture normal and tumoral stem cells from the brain [394] and breast [395], where it is now an established protocol for *in vitro* enrichment and propagation of stem and progenitor cells. More recently, suspension conditions have been used to culture pancreatic progenitor cells [6] and pancreatic cancer stem cells [216]. Indeed, these culture conditions proved to be essential to isolate cells with this behavior since, in monolayers, downregulated expression of the pancreatic progenitor cell markers Ptf1 and Pdx1 as well of the early embryonic enzymes Cpa1 and Ctrb1 and of the stem cell marker Klf4 occurred. Several lines of work indicate that both cell shape and interaction with the extracellular matrix (ECM) [396, 397] can determine the differentiation fate of a cell. In the pancreas it has been shown that maintenance of cell polarity and normal epithelial structure during pancreatic organogenesis is essential for the normal organ development. In the absence of Cdc42, which drives the formation of embryonic pancreatic tubules, the developing pancreas forms large aggregates with no lumen and presents an increase in the number of Cpa1^{+} Ptf1a^{+} progenitors [398]. A similar mechanism may occur in our pancreatic spheres, by which it would be of interest to explore whether inhibition of the pathways regulated by Cdc42, namely the atypical protein kinase C (aPKC), would interfere on sphere formation and/or on acinar cell dedifferentiation.

II. Dedifferentiated acinar cell cultures turn on a senescence program, with activation of the p53 and Ras pathways

Although D5 suspension cultures resemble pancreatic progenitors, they lack important characteristics of these cells, namely the ability to proliferate and to self-renew. In fact, less than 2% of D5 cultured cells express Ki67 or are positive for BrdU incorporation. This observation led us to hypothesize that

dedifferentiated acinar cells activate a senescence program that leads to their irreversible growth arrest. In fact, D5 suspension cultures display senescence associated β-galactosidase (SA β-gal) activity, a hallmark of senescent cells. Furthermore, we found activation of the senescence regulators p53, p21 and Ink4a/p16, and of the senescence marker Dec1. Whole transcriptome analysis of the pathways significantly upregulated in D5 suspension cultures compared with isolated acini, reveals that a p53 mediated response was strongly activated in these cells, as many of the pathways that came out significant were related with downstream targets of this protein, including those related with cell cycle arrest and checkpoint activation (ATM, p53-Hypoxia, p53, ATR-BRCA, G2-CDK1), and with apoptosis (FAS, TNFR1, CASPASE, HIV-NEF, TNFR2). These results and the fact that p21, the main downstream mediator of p53 regulated senescence, is highly activated in D5 cultured acini points out the possible essential role of this protein in the regulation of the growth arrest.

Several factors can induce senescence, such as DNA damage (caused both by dysfunctional telomeres or non-telomeric DNA damage), excessive mitogenic signaling produced by oncogenic stress, or other stress factors, such as perturbations to chromatin organization or oxidative stress [283]. We found no evidence of DNA-damage in our cells, since double immunofluorescence staining for the DNA damage markers phosphorylated histone H2AX (γ-H2AX) and p53-binding protein-1 (53BP1) did not reveal colocalization of these proteins, which typically indicates the formation of senescence associated DNA-damage foci. On the other hand, we detected the activation of Ras and its downstream targets ERK1/2 and, to a lower extent, AKT. Transcriptome pathway analysis also showed increased Ras mRNA levels (data not shown) and activation of MAPK and p38-MAPK signaling pathways. These results suggest that acinar cell isolation and

culture lead to stress, which could be the underlying cause of senescence activation.

We have used several experimental approaches aimed at demonstrating a direct link between Ras pathway activation and the induction of senescence and/or of acinar cell dedifferentiation, namely the culture of acini from conditional triple knockout mice for H, N and K-Ras proteins but we have been unable to completely abolish Ras proteins expression. We have also cultured acinar cells in the presence of ERK inhibitor UO126, which showed a behavior that was similar to that of control cells (data not shown).

Morris et al. have shown that mutant KRas promotes the formation of PDAC precursor lesions in mice by blocking acinar regeneration following acute pancreatitis, through the inhibition of β-catenin stabilization and consequent inhibition of Wnt signaling [210]. Using an *in vitro* model of acinar transdifferentiation to β-cells, Minami et al. reported a role for PI3K, a well established downstream target of Ras, in acinar cell dedifferentiation. These authors have shown that inhibition of PI3K signaling disrupts cell-cell adhesion in acinar cell cultures through the degradation of E-cadherin and β-catenin, maintaining the cells in a dedifferentiated state[399]. These results seem to indicate that activated Ras signaling might in fact be the cause, not only of senescence, but also of the dedifferentiation of pancreatic acinar cells, acting through the destabilization of β-catenin. Further studies, such as the culture of acinar cells from mice with constitutive activation of β-catenin signaling, or the use of inhibitors of the Wnt pathway, would be essential to dissect the role of the β-catenin/Wnt pathway in acinar cell dedifferentiation and its interaction with oncogenic Ras signaling.

III. Acinar cell dedifferentiation and senescence: implications for chronic pancreatitis and PDAC

To evaluate the relevance of our *in vitro* findings to disease, we studied two experimental models of chronic pancreatitis, induced either by ligation of the main pancreatic duct or by treatment with caerulein, a cholecystokinin analogue. The pancreatic duct ligation (PDL) model mimics obstructive pancreatitis in humans and results in the replacement of the normal acinar parenchyma located in the distal part of the ligation by glands with a wide lumen and by ductular structures showing branches and anastomosis. Previous lineage tracing experiments performed using this model have shown that the metaplastic epithelium observed after duct ligation derives from both Ela^+ acinar cells [87] and from pre-existing $Hnf1^+$ ductal cells [44]. The caerulein induced chronic pancreatitis (CaeCP) model displays a more heterogeneous phenotype with most of the acinar glands presenting a wide lumen lined by a monolayer of cells and with few branching ductular complex intermingled. In this model, Strobel et al. have shown that Ela^+ cells give rise to the first type of metaplastic lesions presenting a wide lumen, as well as to mucinous ductal lesions observed after prolonged caerulein treatment, but not to the ductular branching complexes [88].

Analysis of pancreatic marker transcript expression revealed a close parallelism between the changes occurring during acinar cell culture and during induction of chronic pancreatitis in the 2 experimental models. Using immunohistochemistry, we found that the majority of metaplastic cells in the PDL model co-expressed the progenitor markers Pdx1, Ptf1a, Hes1, Sox9, Hnf1b and Foxa2 as well as the ductal keratin Krt19. Similarly, in the caerulein-induced pancreatitis the majority of metaplastic cells co-expressed the progenitor markers Pdx1, Ptf1a, Sox9, Foxa2, and Krt19, while Hes1 displayed cytoplasmic staining and Hnf1b expression was

restricted to the branching tubular complexes. A previous report had already pointed out the temporary reactivation of pancreatic developmental phenotypes upon induction of acute pancreatitis by caerulein which is followed by complete regeneration of the pancreas[123]. Similarly, activation of expression of Pdx1 and Hes1 had also been previously described in a rat model of PDL [86]. Nevertheless, this is the first comprehensive analysis allowing to conclude that cells with pancreatic progenitor features are present in the pancreas after the induction of chronic pancreatitis.

We have also found activation of senescence *in vivo* in the two distinct experimental pancreatitis settings. In the PDL model we observed positive SA β-gal staining, a widespread activation of the senescence markers p53 and Dec1 and a more focal activation of p19/Arf. In the CaeCP model we were unable to find positive SA β-gal staining but we did find a localized activation of p53 and its target p21 as well as a widespread activation of Dec1. The differences between the 2 models are probably due to the fact that in the CaeCP model only an incomplete induction of metaplasia occurs and the pancreas contains areas of normal morphology, while in the PDL model the whole ligated part of the pancreas undergoes metaplasia. Moreover, concomitant with the occurrence of senescence, increased proliferation occurs in pancreatic epithelial cells (12% in the CaeCP model vs. 8% in the PDL model). Hence, *in vivo* two distinct cell populations of cells co-exist in the injured pancreas, one that proliferates and contributes to pancreas regeneration and another one that activates tumor suppressor pathways that lead to an irreversible growth arrest. Concordantly, in the acinar cell cultures we also found heterogeneous expression of the senescence regulators. This suggests that, among cells with an acinar origin, there are two distinct cell populations which present a distinct response to

injury and whose contribution to tumor initiation needs to be evaluated. Whether this responds to the existence of acinar cell heterogeneity also remains to be determined.

Embryonic pancreatic cells are susceptible to malignant transformation by oncogenic *KRas*, since activation of expression of mutant *KRas* during embryonic development gives rise to PanINs and PDAC [91, 156]. By contrast, adult acinar cells are resistant to *Kras*^{G12V} mediated transformation, unless this is combined with the induction of pancreatitis [91]. Our results show that pancreatitis induces a dedifferentiation of adult acinar cells which may render them more susceptible to oncogenic transformation. The activation of senescence observed in normal cells after culture and chronic pancreatitis would function as a tumor suppressive mechanism hampering that dedifferentiated acinar cells give rise to pre-malignant lesions that could progress into carcinoma. In fact, chronic pancreatitis is associated with the occurrence of both ductal complexes and PanINs [400-403] and is a risk factor for PDAC, specially in the case of hereditary forms of the disease[136, 138]. Interestingly, metaplastic lesions associated with pancreatitis harbour mutations in *INK4A* [403, 404] which abrogate one of main pathways regulating senescence. Also, overexpression of p21 and p53 has been observed in human PanINs [405, 406], revealing the activation of senescence in these pre-neoplastic lesions. Moreover, in a mouse model of PDAC induced by oncogenic *Kras* expression, it has been shown that PanINs were positive for senescence markers while PDAC was negative [183]. Altogether, these and our own results indicate that activation of senescence might have an essential role in blocking the progression of dedifferentiated acinar cells into advanced PanINs and later into PDAC. Thus, the abrogation of this pathway through the loss of function of *p16/INK4A* and *TP53*, whose mutations are

very common events in human PDAC [131], would be essential for the development of advanced carcinoma.

Regarding a putative role of the Ras-MAPK pathway in activation of senescence, we did not analyze Ras or its downstream targets in our pancreatitis models. However, previous studies point to the activation of this pathway during experimental pancreatitis. Treatment of rat acini with cholecystokinin, which caerulein mimics functionally, activates Ras and the MEK/ERK pathway [407, 408]. Furthermore, caerulein treatment of rats [409] and murine pancreatic duct ligation [410] were shown to induce the activation of stress kinases in the pancreas, including ERK1/2.

The occurrence of activating mutations in the *KRAS* oncogene is thought to be the primary event occurring in PDAC progression. Our results underscore the fact that, even in the absence of a mutation, increased levels of wild type Ras proteins might have an “oncogenic” effect and promote disease when activated by stress signals, such as the ones present during acinar cell culture or in chronic pancreatitis. In fact, the scarce patients whose PDAC harbors wild type *KRAS* are more likely to have a previous history of chronic pancreatitis [411], pointing to the notion that activation of RAS signaling, even in the absence of mutation, may be essential for PDAC development. In a recent study, Ji et al. have used transgenic mice were mutant *KRas* was expressed at levels much higher than in knockin animals [412]. In these animals, the induction of Ras activity in acinar cells at very high levels resulted in the development of chronic pancreatitis and activation of senescence, supporting our hypothesis that Ras activation can induce both acinar cell dedifferentiation and cellular senescence. In the same experimental model, as the animals aged there was an increase in the formation of PanINs, and at 9 months 50% developed PDAC. Analysis of

tumoral cells from these animals reveled the loss of the tumor suppressors p16 and p15 [412]. These results provide further evidence that abrogation of senescence regulators is a necessary event for the progression of chronic pancreatitis lesions into PDAC.

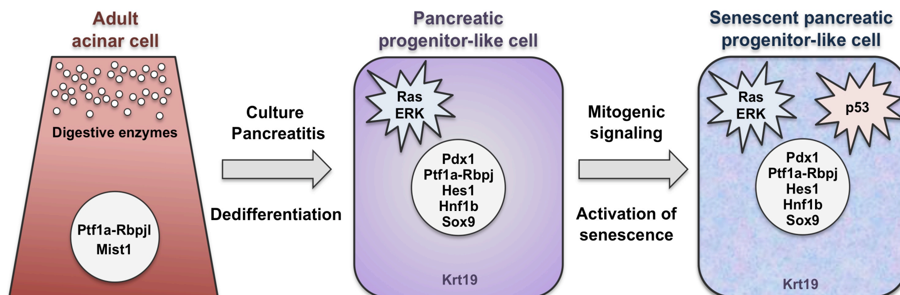


Figure D1. Adult pancreatic acinar cells dedifferentiate to an embryonic pancreatic progenitor phenotype with concomitant activation of a senescence program. Adult acinar cells when subjected to stress, such as cell culture or chronic pancreatitis, undergo dedifferentiation loosing the expression of acinar specific markers such as Rbpjl, Mist1, and the digestive enzymes. Dedifferentiated acinar cells acquire traits of embryonic pancreatic progenitor cells, expressing the transcription factors Pdx1, Ptf1a, Hes1, Hnf1b and Sox9, as well as Keratin 19 (Krt19). Furthermore, they possess the embryonic type PTF1 complex containing Rbpj instead of Rbpjl. Stress induces the activation of Ras-MAPK pathway and consequent mitogenic signaling which is the probable cause for the activation of a senescence program. Senescent pancreatic progenitor-like cells are in a permanent cycle arrest state characterized by strong activation of a p53-mediated response.

IV. Cell cycle arrest in dedifferentiated acini is dependent of p53

Since the two main mechanisms regulating senescence are the p53 and the p16-pRB pathways, which we had found to be activated upon acinar cell dedifferentiation, we took advantage of genetically modified mice to unveil the role of these proteins in the growth arrest observed in the pancreatic progenitor-like cells. Acinar cultures from the pancreas of $p53^{-/-}$ animals

presented an increased proliferation capacity both in suspension and monolayer, revealing the acquisition of resistance to the senescence program activated in wild type cells. Furthermore, $p53^{-/-}$ acinar-derived cultured cells could be expanded for more than 20 passages without showing any signs of growth arrest, suggesting that these cells became spontaneously immortalized. By contrast, acinar cell cultures from $p53^{+/-}$, $p21^{-/-}$ and $p16^{-/-}$ had a behavior similar to wild type controls.

Previous studies have shown that mouse embryonic fibroblasts (MEFs) from p53-null mice do not undergo replicative senescence in normal cell culture conditions and can be propagated indefinitely [267], while MEFs derived from p21-null [413] and p16-null [414] animals activate senescence similar to wild type cells. Moreover senescence induced following coexpression of oncogenic Ras and p53 in MEFs is independent of p21[415]. Therefore, two important conclusions taken from these studies are that: 1) in addition to the p21 mediated response, there are other p53-dependent mechanisms regulating senescence, and 2) these mechanisms are shared in both MEFs and pancreatic epithelial cells, as evidenced by our studies. The results described here are also in agreement with the idea that the p53 pathway plays a more prominent role than the p16 pathway in regulating senescence in mouse cells [265]. Furthermore, Bardeesy and colleagues observed, using PDAC mouse models, that animals expressing oncogenic *Kras* in combination with p53 loss developed fast progressing tumors, with a shorter latency than those animals with p16 loss [191]. Altogether, these observations suggest that the p53 pathway has a more potent effect as a tumor suppressor in murine pancreatic acinar cells than the p16-Rb pathway. Nevertheless, it is known that both the regulation of senescence and cellular transformation result from the integration of several mechanisms and the contribution of different pathways varies among cell

types and species [416]. Furthermore, in human tumors *p16/INK4A* loss seems to precede *TP53* loss during PDAC progression [131] by which it is plausible that in human acinar cells, inactivation of both pathways is necessary for the abrogation of senescence and consequent development of invasive carcinoma.

V. Lack of p53 induces an EMT in pancreatic epithelial cells

Characterization of the phenotype of subcultured $p53^{-/-}$ cells revealed that only after 2 passages in suspension these cells activated the expression of the mesenchymal marker vimentin and lost expression of E-cadherin in most cells. To demonstrate the epithelial origin of the vimentin-expressing cells, we performed lineage tracing by culturing acinar cells from $Ptf1a^{Cre}$; R26R-EYFP; $p53^{-/-}$ animals in which all pancreatic epithelial cells are $EYFP^{+}$. This also allowed us to separate the progeny of pancreatic epithelial cells by FACS from contaminating $EYFP^{-}$ mesenchymal cells. Immunofluorescence analysis performed on passage 12 (P12) YFP^{+} cells showed that only 5% of cells retained Krt19 expression while all cells presented strong expression of vimentin, clearly demonstrating the occurrence of an epithelial-mesenchymal transition (EMT) in pancreatic epithelial-derived cells. Immunofluorescence using P12 cells revealed that E-cadherin expression was generally absent and, when present, it was expressed at very low levels and accumulated intracellularly, being absent from the cell-cell contacts. Analysis of early passages demonstrated an increased expression of the mRNA of the EMT regulators Twist, Snai1, Snai2, Zeb1 and Zeb2 in $p53^{-/-}$ D5 suspension cultures. After dissociation, sorting and expansion of $p53^{-/-}$ YFP^{+} cells, we observed a sharp upregulation in the expression of these transcription factors and of vimentin, and a strong downregulation of E-

cadherin that was evident already on P1, revealing that the activation of the EMT occurs very fast upon primary acinar cell culture.

The lineage tracing system used in these experiments does not allow to distinguish between different pancreatic epithelial cells, by which we cannot formally rule out that cells undergoing EMT can originate from ductal or endocrine cells. Nevertheless, our previous studies have shown that acinar cells constitute more than 93% of our initial cell preparation and lineage tracing experiments using acinar cells from $Ela^{CreERT2}$;R26R-LSL-LacZ mice demonstrated that the cells forming the primary spheres have an Ela^{+} origin, by which acinar cells are the strongest candidate to constitute the epithelial compartment having undergone an EMT. To definitely answer this question, $Ela^{CreERT2}$; R26-EYFP; $p53^{lox/lox}$ crosses are being established which will allow us to trace cells of acinar origin and to knockout the expression of p53 specifically in the acinar compartment, in a time controlled manner. Similarly, experiments to analyze the fate of endocrine cells are ongoing in the laboratory.

Our work highlights a new function of p53 in the maintenance of the epithelial differentiated phenotype and, for the first time, we show a wild type p53-dependent EMT in normal pancreatic cells. Previous studies had evidenced a gain of function of mutant p53 in promoting the acquisition of mesenchymal characteristics with consequent increased migration and invasion in breast cancer cells [298, 299], and in inducing the EMT regulator Twist in immortalized prostate cells [417]. More recently, $p53^{R175H}$ was shown to induce invasion of pancreatic tumor cells and to promote metastasis in a mouse model of PDAC [308]. Our work independently provides evidence for an important role of p53 in this process that was unanticipated at the initiation of the study. Very few studies had evidenced a role of wild type p53 in EMT

until very recently. Snai2/Slug has been shown to be a direct target of p53 in the hematopoietic system [418] and wild type p53 has been shown to induce Slug degradation through the formation of a p53-Mdm2-Slug complex [300]. As this thesis report was being written, a study was published demonstrating that wild type p53 can directly bind to the promoter of the microRNA miR-200c and activate its expression, inducing the downregulation of its targets Zeb1 and Zeb2 and promoting an epithelial phenotype. Downregulation of p53 in mammary epithelial cells leads to a decreased expression of miR-200c and activates the EMT program [419]. This work provides a mechanism through which p53 can regulate EMT; whether this mechanism is responsible for the observed effects in pancreatic cells is currently being analyzed in our laboratory. Moreover, we are exploring additional mechanisms through which these effects may take place.

Another interesting finding is the fact that, in D5 suspension cultures, p53$^{+/-}$ cells show an upregulation of the expression of mRNAs for EMT regulators. By contrast, p53$^{+/-}$ cells do not display unlimited proliferation thus clearly distinguishing these two phenotypic effects. Chang et al. have also shown that downregulation of p53, rather than its inactivation, is sufficient to induce an EMT [419], indicating that different thresholds of p53 levels control different roles of this protein in cells. Furthermore, it highlights the importance of p53 allelic loss in tumor development, indicating that cells with a single allele of p53 can be more prone to acquire mesenchymal properties and, consequently, increased migration and invasion. In fact, it has been shown that allelic loss precedes the mutational event in the biallelic inactivation of *TP53* during PDAC progression [420]. Furthermore, p53$^{+/-}$ mice develop tumors much earlier than wild type animals, and a high proportion of the tumors from p53$^{+/-}$ mice retain an intact, functional, wild

type *Trp53* allele, showing p53 haploinsufficiency in the promotion of tumorigenesis [421].

VI. Pancreatic acinar cell cultures lacking p53 have increased stemness properties

Characterization of primary acinar cell cultures showed that, in the absence of p53, cells present increased stemness properties, evidenced by an increase in sphere formation capacity and an upregulation of pancreatic progenitor and endoderm markers, as well of the stem cell genes c-myc, Bmi1, Klf4, Cd133 and Abcg2. Upon dissociation and expansion, these cells lost the pancreatic progenitor phenotype but retained the expression of endoderm and stem cell markers concurrently with the activation of mesenchymal properties. These results indicate that the loss of p53 allowed further dedifferentiation of pancreatic acinar cells, which loose the pancreatic progenitor phenotype, acquiring features of pre-pancreatic endoderm, evidenced by the increased expression of Hhex. It remains to be established whether indeed pre-pancreatic endodermal cells in the embryo express any mesenchymal markers, as might be suggested by our work.

Several very recent studies have brought up the idea that besides its known tumor suppressor functions, p53 also has an important role in stem cell biology. Previous work had demonstrated a role of p53 in reducing the pluripotency of ESC since activated p53 suppresses Nanog expression and induces ESC differentiation [332]. Several studies have also shown that p53 constitutes a barrier for the reprogramming of somatic cells into induced pluripotent stem (iPS) cells, as p53-null cells present a remarkable increase in the reprogramming frequency [338-341]. Furthermore, several studies have shown that p53 controls the self-renewal capacity in adult multipotent cell populations, namely in neural [343], hematopoietic [345, 346] and mammary stem

cells [344, 345]. Here, we show for the first time that p53 also controls cell plasticity in the adult pancreas, by constituting a barrier to the reprogramming of pancreatic acinar cells and to the acquisition of increased stem cell properties.

We did not explore the mechanism linking loss of p53 and stemness. However, previous work in the mammary system has highlighted some of the mechanisms involved therein. Studies performed on mammospheres demonstrated that p53 regulates the polarity of stem cell division and its depletion results in an increased number of self-renewing divisions [347], and that p53 can restrict the stem cell potential through the activation of the Notch pathway [348]. Also in mammary cells, it has been shown that the EMT process can generate cells with increased stem cell properties [377, 378]. The work by Chan et al. also demonstrates a link between p53 and the regulation of both EMT and stemness, through its direct regulation of miR-200c. p53 downregulation and consequent decrease in miR-200c results in an upregulation of Klf4 and Bmi1, two stemness-associated genes that are known RNA targets of miR-200c, and in an increase in the $Cd24^{-}$ $Cd44^{+}$ stem cell population [419]. In our system, the same stem cell markers are upregulated in the absence of p53 in association with EMT, suggesting that similar mechanisms are involved in pancreatic cells.

One aspect of our work that needs to be extended is the analysis of the differentiation potential of the immortalized p53-null cells. For this, we plan to undertake different approaches: to induce their differentiation *in vitro*, by culturing the dedifferentiated cells with different cocktails of signaling factors and through co-culture with pancreatic embryonic explants, and *in vivo*, by injecting the cells into the kidney capsule of immunodeficient mice.

VII. p53-null mice have a delay in acinar cell regeneration and increased acinar cell proliferation upon induction of injury

In vivo experiments revealed that under normal conditions, acinar cells from $p53^{-/-}$ animals present no alterations in histology or proliferation, being similar to wild type acinar cells. The same is true for other pancreatic cell types, by which p53-null pancreas has a completely normal histological appearance. Regarding the expression of transcripts of acinar, pancreatic progenitor or epithelial markers, p53-null pancreata also presented no differences from controls and the levels of acinar enzymes in blood were normal. These results seem to indicate that in the absence of stress, lack of p53 does not have any affect in pancreatic acinar cell growth or differentiation. Nevertheless, p53 null animals present a reduced pancreatic expression of some EMT transcription factors. These differences may be due to the fact that these animals are total knockouts for p53 by which not only the epithelial but also other cells constituting the pancreas may be affected.

Preliminary experiments to analyze the *in vivo* effect of pancreatic injury induction revealed that, 48h after the induction of caerulein damage, pancreata from both wild type and $p53^{-/-}$ mice presented a similar histological response and developed alterations characteristic of acute pancreatitis, including increased edema, the presence of inflammatory cell infiltrates, vacuolization of acini, and formation of tubular complexes. At 48h, p53-null mice presented a moderate increase in acini vacuolization, but the differences were not significant. Eight days after the initiation of the caerulein acute treatment, wild type animals had partially recovered from the acute pancreatitis, while $p53^{-/-}$ animals presented larger areas of ductal metaplasia, as indicated by an increased score in acini vacuolization and tubular complex formation, suggesting that in the absence of p53, there is a

delay in acinar cell regeneration. Nevertheless, probably due to the reduced number of animals, these differences failed to reach statistical significance. In a future experiment it will be important to analyze a larger number of animals and to include a later time point to determine whether p53-null animals are able to undergo complete acinar cell regeneration.

Analysis of proliferation at 48h demonstrated that, while all the other pancreatic cell compartments behaved similarly in $p53^{-/-}$ and in wild type control animals, $p53^{-/-}$ acinar cells presented a 6-fold increase in the number of $Ki67^{+}$ cells. Thus, similarly to our *in vitro* system, p53-dependent mechanisms control the growth of acinar cells under stress conditions *in vivo* in the adult pancreas. Eight days after caerulein treatment, none of the cellular compartments in the $p53^{-/-}$ pancreas, including acini, presented significant differences in cell proliferation. These results indicate that additional p53-independent mechanisms contribute to the regulation of acinar cell growth *in vivo*, blocking at later time points the increased acinar cell proliferation observed after damage. Since the p16-Rb pathway is the other important regulator of senescence [283] and both *p16/INK4A* and *TP53* are commonly mutated in PDAC [131], it would be of interest to analyze the status of p16 activation in our model, as well as the response to pancreatitis in animals lacking both p16 and p53 tumor suppressors.

Regardless of the effect in acinar cell proliferation 48h after caerulein treatment initiation, the expression of acinar genes in the pancreas and the level of acinar enzymes in the blood of $p53^{-/-}$ mice was similar to that of controls. Also, the levels of transcripts for pancreatic progenitor, epithelial and mesenchymal markers in the pancreas of $p53^{-/-}$ animals presented no statistically significant differences from wild types. Similarly, at day 8 we did not find any effect on pancreatic gene expression in p53 knockouts, apart

from decreased levels of pancreatic mRNA expression of some mesenchymal cells markers, similarly to what had been observed in untreated animals. These results indicate that no increased acinar cell dedifferentiation or EMT occurred *in vivo* after caerulein-induced acute pancreatitis.

Altogether, our preliminary *in vivo* results suggest that the p53-dependent effects on acinar cell proliferation are independent from the regulation of EMT and of stemness, as we did not observed the occurrence of these last two traits after induction of acute pancreatitis. Thus, additional p53-independent mechanisms exist that control the plasticity of acinar cells in the adult pancreas. Yet, one must consider that while the *in vitro* system constitutes a simplified model where isolated acinar cells are cultured in a defined medium allowing us to control the environment and to dissect cell-specific mechanisms, in the pancreas, acinar cells have to integrate distinct signals from the niche where they are inserted which include other pancreatic cellular types, stromal cells and the vascular system, by which non-autonomous mechanisms may also have a role in controlling pancreatic acinar cell differentiation.

The acute model used in this study does not inflict a severe damage to the pancreas and it would be worthwhile to explore other strategies for induction of pancreatitis in $p53^{-/-}$ mice, including prolonged treatment, multiple bouts of acute pancreatitis, use of arginine-induced pancreatitis, or pancreatic duct ligation (PDL). A previous study using the PDL model evidenced a role for p53 in acinar cell apoptosis, and a reduced formation of tubular complexes in the absence of p53 [422], but additional analyses are required as these authors did not evaluate acinar cell dedifferentiation or the occurrence of EMT and increased stem cell traits.

Moreover, we have used constitute $p53^{-/-}$ mice and therefore cannot distinguish the effect of the lack of p53 on acinar cells from the effect on other cellular compartments, including the stroma. In fact, it has been previously shown that p53 has a role in the control of the inflammatory response in mice [423, 424]. Thus, in future experiments we plan to use $Ela^{CreERT2}$; R26-EYFP; $p53^{lox/lox}$ animals, in which we will be able to eliminate p53 selectively in acinar cells and which, in addition, will allow us to trace the fate of acinar cells and truly evaluate the occurrence of EMT in the injured pancreas.

VIII. p53: a barrier to pancreatic acinar cell plasticity and to PDAC initiation

In the absence of p53, pancreatic acinar cultures concomitantly become resistant to senescence, undergo EMT, and gain increased stem cell properties. Interestingly, previous studies have evidenced that these traits not only are related with each other but also and very importantly, provide a link with carcinogenesis. The EMT is a crucial process in tumor progression and dissemination since it provides cells with increased migration and invasion properties and it has also been related with the acquisition of resistance to chemotherapy [350]. Furthermore, transcription factors involved in EMT have been associated with increased resistance to apoptosis and to senescence. Twist1 inhibits the ARF-p53 pathway, restraining p53-mediated apoptosis [425, 426], and both Twist1 and Twist2 prevent oncogene-induced senescence through the inhibition of p16 and p21 [427]. Similarly, Zeb1 inhibits MEFs premature senescence and in Zeb1 mutant MEFs there is ectopic expression of p15 and p21 [428]. Interestingly, EMT regulators have also been shown to increase stem cell properties in cancer cells. Zeb1 was shown to increase the tumor-initiating capacity of pancreatic and colorectal

cancer cells. Zeb1 acts by repressing the expression of the miR-200 family, thereby activating the expression of their target Bmi1 [381]. Twist also regulates the expression of Bmi1 and co-expression of both proteins is essential to promote EMT and the tumor-initiating capacity in head and neck squamous cell carcinoma [380].

By blocking both cell cycle progression and the epithelial phenotype of pancreatic acinar cells, p53 provides a link between pancreatic stem/progenitor cells and cancer. The activation of p53 in pancreatic acinar cells subjected to stress might control not only the proliferation of dedifferentiated acini but, more importantly, it might suppress the generation of cells with increased migration potential and stem cell properties. In view of the cancer stem cell model, the loss of p53 may favor the generation of cancer initiating cells with increased metastatic potential. In fact, many lines of evidence point to a link between p53 and increased tumoral invasion. In human breast cancer xenografts, abrogation of p53 leads to the activation of a molecular signature that is associated with cell proliferation, EMT and survival, and with the development of a more invasive and metastatic phenotype [429]. Very recent work in a mouse model of colorectal cancer has also indentified a set of genes controlled by p53 that are associated with increased invasiveness of tumoral cells [430]. In a mouse model of pancreatic cancer generated by viral delivery of the polyoma virus middle T antigen (PyMT), deletion of *Trp53* led to the generation of more metastatic tumors [431]. Moreover, in human PDAC, *TP53* mutations have been associated with increased metastasis [432] and poor survival [432, 433]. Bearing in mind this panorama, it is conceivable that the novel function of p53 unveiled by this study as a protector of pancreatic acinar cells plasticity may in fact constitute a key tumor suppression

function in the injured pancreas, preventing the initiation of invasive carcinoma.

Based on the biological importance of EMT and its associated stem cell properties, a reversal of this phenotype could provide a potentially attractive therapeutic approach. Chang et al. reported that overexpression of wild type p53 or miR-200c in mammary cells that had undergone EMT were able to revert the mesenchymal phenotype and to diminish the stem cell population [419]. Our own preliminary experiments indicate that p53-null pancreatic acinar cultures with a mesenchymal phenotype are able to form epithelial cysts when cultured in a 3D matrigel matrix (Annex Figure), suggesting a role of the ECM in the process. As future work, we aim at better characterizing these cysts and the cellular population giving rise to them, as well as exploring which components of the ECM promote the re-establishment of the epithelial phenotype. Furthermore, we plan to re-express p53 in pancreatic acinar cultures taking advantage of the p53-ER system [434], which will allow us to control the time and level of p53 activation. With this system we will analyze whether a MET takes place and we will explore the threshold at which p53 controls different pathways and biological functions in normal pancreatic epithelial cells. A better understanding of the mechanisms through which p53 acts as a tumor suppressor in pancreatic cells may help the development of strategies based on the reactivation of this pathway, aiming to restrain the development and dissemination of PDAC.

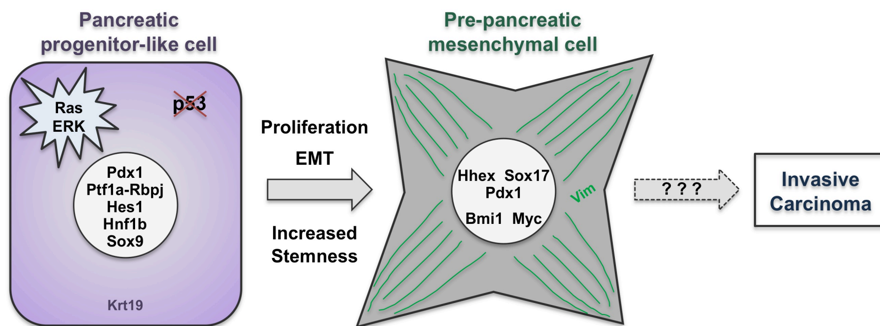


Figure D2. p53-dependent regulation of growth, EMT and stemness in pancreatic epithelial cells. Dedifferentiated pancreatic epithelial cells lacking p53 expression undergo a bypass of cell cycle arrest, being able to proliferate and becoming immortalized. p53-null cells activate an EMT programme loosing the expression of Krt19 and gaining the expression of mesenchymal markers such as vimentin (Vim). Cells with mesenchymal characteristics loose Ptf1a and express markers of pre-pancreatic endoderm such as Hhex, Sox17 and Pdx1 in combination with stem cells markers as Bmi1 and c-myc. Therefore, the loss of p53 in pancreatic epithelial cells can lead to the generation of cells with increased migration potential and stem cell properties, that might be involved in the initiation and dissemination of invasive pancreatic carcinoma.

CONCLUSIONS

Conclusions

1. Adult pancreatic acinar cells cultured in suspension dedifferentiate into embryonic pancreatic progenitor-like cells.

2. Pancreatic acinar cell cultures undergo growth arrest, turning on a senescence program with strong activation of the p53 and Ras-MAPK pathways.

3. Dedifferentiated acinar cells and senescent cells can be found *in vivo* in two distinct murine models of experimental chronic pancreatitis indicating the relevance of the *in vitro* findings to pathophysiology of disease.

4. In the absence of p53, but not of p21 or p16, dedifferentiated acinar cultures bypass cell cycle arrest and become immortalized.

5. p53-null pancreatic cells undergo a rapid epithelial-mesenchymal transition. These cells maintain the expression of pre-pancreatic endoderm and stem cell markers.

6. In an experimental model of caerulein-induced acute pancreatitis, lack of p53 leads to a delay in acinar cell regeneration and is associated with increased acinar cell proliferation.

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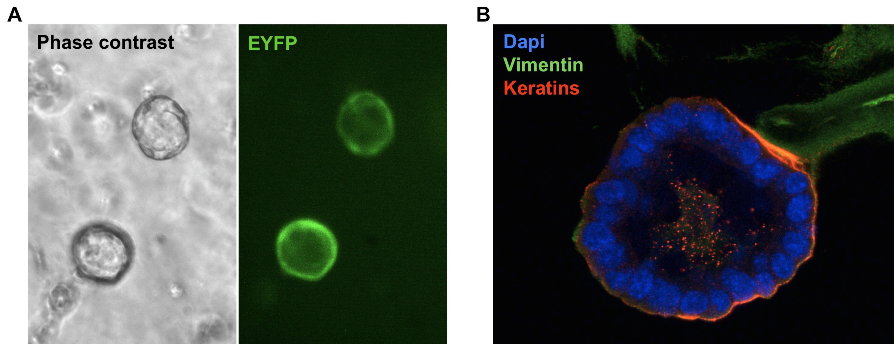
ANNEX

Preliminary Results



Annex Figure. Acinar cultures from p53$^{-/-}$ mice which had undergone EMT reacquire an epithelial phenotype in 3D culture. Acinar cell cultures from Ptf1a^{Cre};R26R; p53$^{-/-}$ mice, which had been kept in monolayer culture with defined medium for 20 passages, where cultured embedded in a 3D matrix of Matrigel (BD Biosciences) in the presence of RPMI medium supplemented with 10% FBS. **A.** EYFP+ cells form cysts in 3D culture. **B.** Immunofluorescence staining for the mesenchymal marker vimentin and for epithelial keratins (Pan-keratin antibody) shows that cells forming the cysts reacquire an epithelial phenotype.