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Yajie Zhao

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Yajie ZHAO

Etude du rôle des voies de signalisation des Activines dans l'initiation et la progression du cancer du pancréas

---

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RÉSUMÉ DE LA THÈSE

L'adénocarcinome canalaire du pancréas (PDAC) est un cancer agressif qui représente la 7ème cause de décès par cancer au cours des 5 dernières années. L'absence de marqueurs permettant la détection de ce cancer à un stade précoce, combinée à la faible efficacité des traitements actuels, conduit fréquemment à son diagnostic tardif où les patients ont déjà développé des lésions incurables. Comprendre les mécanismes moléculaires impliqué dans l'initiation des lésions néoplasiques pancreatiques est donc crucial pour espérer améliorer la prise en charge clinique et thérapeutique des patients atteints de PDAC. Mon sujet de thèse visait à comprendre les conséquences fonctionnelles des mutations rapportées pour ALK4, un récepteur de la famille TGFβ, dans les mécanismes d'initiation du cancer du pancréas. Mes travaux rapportent l'identification de l'ActivinA comme le ligand majeur d'Alk4 dans les lésions métaplasiques canalaires (ADM) et Néoplasiques Intraépithéliales Pancréatiques (PanINs). Ils soulignent que l'ActivinA est produite par les cellules sénescentes retrouvées dans les lésions néoplasiques en réponse au stress-oncogénique induit par Kras. Mes resultats identifient l'ActivinA comme un facteur bénéfique du secretômes produit par les cellules sénescentes (SASP) qui contribue à limiter la prolifération et la progression des lésions ADMs et PanINs. Des travaux complémentaires suggèrent que la signalisation de l'ActivinA/Alk4 contribuerait également à maintenir l'expression de Dclk1 (un marqueur souche) dans les cellules sénescentes indifférenciées retrouvées au sein des PanINs et a de contribuer via ce processus au remodelage de l'environnement tumoral. Dans leur ensemble, mes travaux soutiennent le rôle pro-sénescent et protecteur de l'ActivinA et de sa signalisation via Alk4 dans les lésions pancréatiques néoplasiques. Ils suggèrent également que l'utilisation de thérapies ciblant l'ActivinA devraient toutefois être envisagée avec prudence car elles pourraient favoriser l'apparition de lésions pancréatiques pré-néoplasiques.

ABSTRACTS

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer that represents the 7th cause of cancer related death over the past 5 years. The lacks of specific tools and markers to detect PDAC at an early stage combined with the poor efficiency of curative treatments lead to a frequent late diagnostic when patients have already develop advanced incurable PDAC lesions that results in a high mortality. Understanding the molecular mechanism that drives PDAC onset in its early stage is therefore of great importance to improve the clinical outcome for PDAC patients. The aim of my PhD was to understand the functional consequence of the mutations reported for the TGFβ-related ALK4 receptor in PDAC with a focused interest in the mechanisms that are driving the early onset of pancreatic cancer. Using data-mining and relevant mouse model combined with genetic and chemical knock-down strategies, my work identified ActivinA as the main ligand that signals through Alk4 in Acinar to Ductal metaplastic (ADMs) and pancreatic intraepithelial neoplastic (PanINs) lesions. More importantly, my work underlines that ActivinA acts as a beneficial SASP factor, induced and produced by ADM subjected to Kras-Oncogenic Induced Senescence (OIS), that functions through Alk4 to limit the proliferation and progression of PanINs. Complementary work revealed that ActivinA-Alk4 signalling contributes to sustain the expression of the Dclk1 tuft-cell marker in the undifferentiated duct senescent-cells that maintains within PanINs, a cell population that my preliminary data suggests to contribute to the remodelling of the tumour stroma. Taken together my PhD work supports the pro-senescent and protective role of ActivinA-signalling through Alk4 during ADM and its potent contribution in remodelling of the pancreatic environment. It further suggests that the use of ActivinA-signalling targeting therapeutics should be considered with caution as it may contribute to favour the onset of preneoplastic pancreatic lesions.

Key words: Pancreatic cancer, TGFβ/Activin signalling, Acinar-to-Ductal Metaplasia, Oncogene induced senescence, SASP, Dclk1

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LIST OF ABBREVIATIONS

ADEX: aberrantly differentiated endocrine exocrine

ADM: acinar-to-ductal metaplasia

ACVR1B: activin receptor type 1B

AER: apical ectodermal ridge

ALK: Activin Receptor-like kinase

AMY2: alpha amylase

AP-1: activator protein 1

ARF: Alternate-reading-frame

α SMA: α-smooth muscle actin

ATM: Ataxia-telangiectasia-mutated

ATR: Ataxia telangiectasia and Rad3-related

bHLH: basic helix-loop-helix

BMPs: bone morphogenic proteins

BRAF: v-Raf murine sarcoma viral oncogene homolog B1 gene

BRD4: bromodomain-containing protein 4

BrdU: Bromodeoxyuridine

Brg1: Brahma-related gene 1

CA 19-9: carbohydrate antigen 19-9

CAFs: cancer-associated fibroblasts

CCK: cholecystokinin

CDKN2A: cyclin-dependent kinase inhibitor 2A

CEBPβ: CCAAT/enhancer binding protein-β

CELA1: chrymotrypsin-like elastase 1

CHK2: Checkpoint kinase 2

CK19: cytokeratin 19

CM: conditioned medium

CPA1: carboxypeptidase A1

CT: computed tomography

CTRB1: chymotrypsinogen B1

CXCL: C-X-C chemokine ligand

CXCR: C-X-C chemokine receptor

Dclk1: doublecortin-like kinase 1

DcR2: Decoy receptor 2

DDR: DNA damage response

DEC1: Deleted in Esophageal

ECM: Extracellular matrix proteins

EGFR: epidermal growth factor receptor

EGR1: early growth response protein 1

ELA1: elastase 1

EMT: epithelial-to –mesenchymal transition

ERCP: endoscopic retrograde cholangiopancreatography

EUS: endoscopic ultrasound

FSH: follicle stimulating hormone

GATA4: GATA binding protein 4

GDFs: growth and differentiation factors

GEMMs: genetically engineered mouse models

GFR: glomerular filtration rate

GLB1: Galactosidase beta 1

GM-CSF: granulocyte-macrophage colony-stimulating factor

hCAP-18/LL-37: cationic antimicrobial peptide 18/LL-37

γ -H2AX: H2A histone family member X

H3K9me3: Trimethylation at lysine 9 of histone 3

HGF: hepatocyte growth factor

HMGB2: high mobility group box 2

HP1γ: Heterochromain protein 1 homologue-γ

HSCs: hepatic stellate cells

IL: interleukin

IPF: idiopathic pulmonary fibrosis

IPMNs: intraductal papillary mucinous neoplasms

I-smads: inhibitory Smads

KLF4: Krüppel-like factor 4

Kras: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

LAP: latency associated peptide

LKB1: liver kinase B1

LMNB1: Lamin B1

MCNs: mucinous cystic neoplasms

MCP: monocyte chemoattractant proteins

MIP: macrophage inflammatory proteins

MLL1: mixed-lineage leukemia 1

MMPs: matrix metalloproteinases

MR: magnetic resonance

MRCP: magnetic resonance cholangiopancreatography

NCCN: National Comprehensive Cancer Network

NF1: neuroflibromin

NFATC: nuclear factor of activated T cells

NF- κ B: nuclear factor κ B

OIS: oncogene-induced senescence

PAI-1: plasminogen activator inhinibor 1

PanINs: pancreatic intraepithelial neoplasias

PanNET: pancreatic neuroendocrine tumors

PCSCs: pancreatic cancer stem cells

PDAC: pancreatic ductal adenocarcinoma

PDEC: pancreatic duct epithelium

PDG: pancreatic duct glands

PDGF-AA: platelet-derived growth factor AA

PDX1: pancreatic and duodenal homeobox 1

PET: positron emission tomography

PI3K: phosphatidylinositol 3-kinase

PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase

PMK: primary mouse keratinocytes

PRKD1: serine/threonine-protein kinase D1

PTEN: phosphatase and Tensin homolog

PTF1: pancreas transcription factor 1 complex

Ptf1a/p48: Pancreas Associated Transcription Factor 1a

PSCs: pancreatic stellate cells

Rb: Retinoblastoma

RBPJ: recombining binding protein suppressor of hairless

ROS: reactive oxygen species

R-smads: receptor-activated smads

SA-β-Gal: senescence-associated β-galactosidase

SAHF: Senescence-associated heterochromatic foci

SARA: Smad anchor for receptor activation

SAS: senescence-associated stemness

SASP: senescence-associated secretory phenotype

SBE: Smad Binding Element

SBRT: stereotactic body radiotherapy

SMAD4/DPC4: SMAD family member 4

SMS: senescence messaging secretome

SOX9: SRY-related HMG box factor 9

SPRY2: Sprouty2

STAT3: signal transducer and activator of transcription 3

TBX3: T-box transcription factor 3

TGF α : transforming growth factor α

TGF β : transforming growth factor beta

TGF-β1: transforming growth factor beta 1

TIMP: Tissue inhibitor of metalloproteinases

TMA: tumour-associated macrophages

Twist1: Twist-related protein 1

VEGF: vascular endothelial growth factor

VHL: von Hippel-Lindau disease tumor suppressor

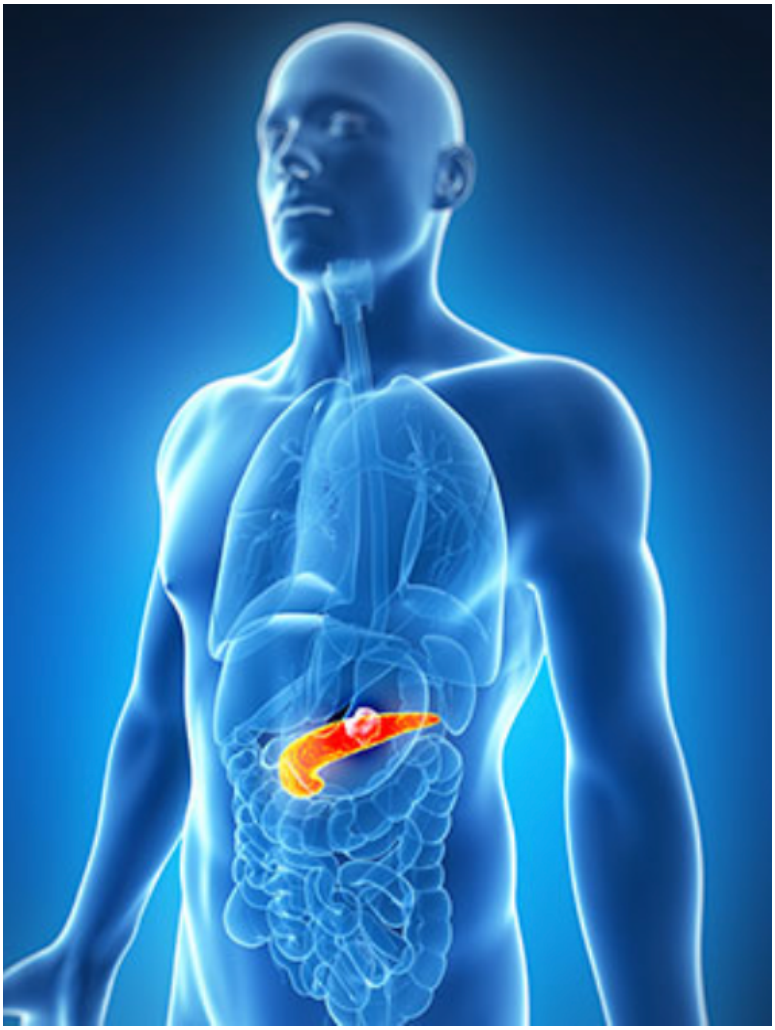
VSMCs: vascular smooth muscle cells

WNT16B: Wingless-type MMTV integration site family member 16B

X-gal: chromogenic substrate 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside

Once a upon a time, a story about pancreas.....

(Picture from https://www.pancan.org/facing-pancreatic-cancer/about-pancreatic-cancer/what-is-pancreatic-cancer/)



INTRODUCTION

1 Pancreatic cancer

Pancreatic cancer is one of the deadliest cancer worldwide, representing the 7th leading cause of cancer-related death over the past 5 years. Data from GLOBOCAN 2018 which includes 185 countries and 36 cancers estimate that pancreatic cancer contributes to 2.5% of new cancer cases and 4.5% of cancer deaths each year (Bray *et al.*, 2018). Current progression trend should lead to an important increase of pancreatic cancer incidence (+77.7% with 356,358 new cases) and mortality (+79.9% with 345,181 deaths) by 2040 (Bray *et al.*, 2018). Risk factors such as tobacco smoking, diabetes mellitus, obesity, dietary factors, alcohol abuse, age, ethnicity, family history, genetic factors, *Helicobacter pylori* infection, non-O blood group and chronic pancreatitis, have shown existing correlations with pancreatic cancer incidence and mortality yet the exact reasons of such association remain to be fully ellucidated (Midha *et al.*, 2016). **Pancreatic ductal adenocarcinoma (called PDAC)** is the most common type of all pancreatic cancer. Patients usually have a poor survival chance after diagnosis (24% survive for 1 year and 9% live for 5 years) due to the lack of sensitive and specific detection tools in the early disease stage and a poor efficiency of curative treatments such as surgical resection and adjuvant therapy (Stewart and Wild, 2018). As a consequence, there is an urgent need to better understand the molecular pathology especially at the early stage of this lethal disease in order to improve and develop novel diagnostic and therapeutic strategies.

In this chapter on Pancreatic Cancer, I will first introduce their classification and provide details about their clinical stages, including recent work on their gene profile grouping. The second section will present their clinical features and will provide general informations about patients diagnostic and treatments. A third section will summarize our current knowledge regarding their cellular origin, histological evolution and recent understanding on acinar metaplasia as a mechanism of PDAC initiation.

1.1 Pancreatic cancer classification and clinical stages

The pancreas is an elongated and pear-shaped gland located deep in the abdomen, behind the stomach [Figure 1]. It is made up of two major components: the exocrine component which is made up of ducts and acinus produces enzymes for digestion, while the endocrine component which consists of islets of Langerhans makes specific hormones (most importantly insulin) that play an important role in regulating the body's metabolism.

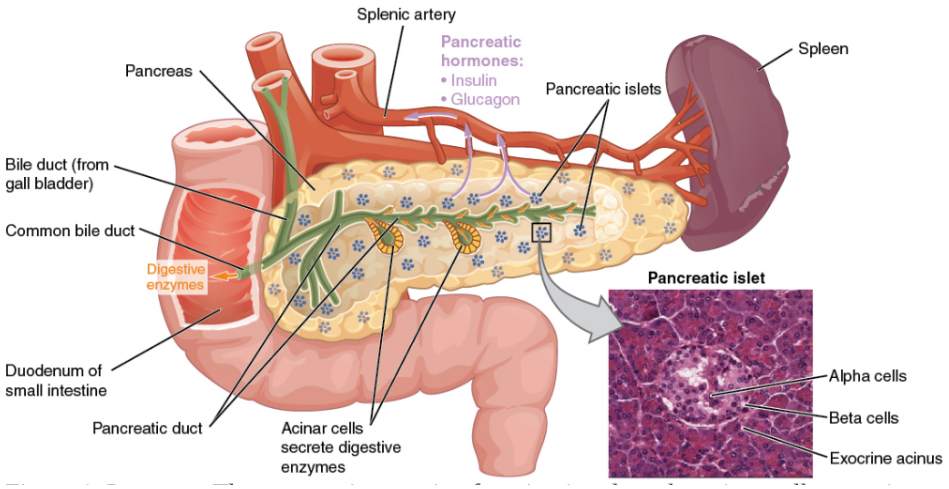


Figure 1. The anatomy of pancreas. (Pictures from BC campus Chapter 17. The Endocrine System and mocrograph provided by the Regents of University of Michigan Medical School © 2012)

- Histological/grade classification

Pancreatic cancer is usually grouped into two main types: exocrine tumors and endocrine tumors. Exocrine tumors are the most common form of pancreatic cancer, account for over 95% of all pancreatic cancers (called PDAC). It is the most common type, making up about 90% of all exocrine tumors (Hidalgo *et al.*, 2015). Other types of exocrine tumors are rare. Endocrine tumors also called pancreatic neuroendocrine tumors (PanNET) or islet cell tumors which is less common than exocrine tumors (less than 5%) include insulinoma, glucagonoma, gastrinoma, somatostatinoma, VIPomas and PPomas.

Pancreatic adenocarcinoma staging is based on tumor size, location within the pancreas, involvement of surrounding vessels, and presence of metastasis. In the United States, the common used staging system is defined from the American Joint Committee on

Cancer and the National Comprehensive Cancer Network. Thus, pancreatic cancers are classified into the four following types based on the clinical stage of the tumor:

Type I with no tumor spread or being resectable;

Type II with local spread or borderline resectable;

Type III with wide spread or being unresectable;

Type IV with metastatic lesions (Chun *et al.*, 2018).

- Classification based on gene profiling

In recent years a novel classification based on gene profiling and integrated analysis of large genomic studies has emerged. One representative study is from Bailey's *et al.*, in which those authors took advantage of an integrated genomic analysis of 456 pancreatic ductal adenocarcinomas with 32 recurrently mutated genes that aggregate into 10 pathways: *KRAS, TGF-β, WNT, NOTCH, ROBO/SLIT* signalling, *G1/S* transition, *SWI-SNF*, chromatin modification, DNA repair and RNA processing (Bailey *et al.*, 2016) [Figure 2]. Interestingly, while *KRAS* accounts for 92% of total activating mutations, members within ***TGF-β* signalling** (*SMAD4, SMAD3, TGFBR1, TGFBR2,* ***ACVR1B*** *and ACVR2A*) also harbor a 47% mutation. Moreover, the role of **activin signalling pathway** was highlighted [Figure 2]. They further defined four subtypes based on the differential expression of crucial transcription factors and downstream targets in lineage specification and differentiation during development and regeneration of pancreas, including: I) squamous; II) pancreatic progenitor; III) immunogenic; and IV) aberrantly differentiated endocrine exocrine (ADEX).

The Cancer Genome Atlas Research Network later validated approaches for accurate genomic profiling on samples with low neoplastic cellularity that have been underrepresented in previous genome sequencing efforts (Cancer Genome Atlas Research Network, 2017).

Taken together, these new classification provide new insights in the molecular evolution of pancreatic cancer subtypes and opportunities for precision medicine.

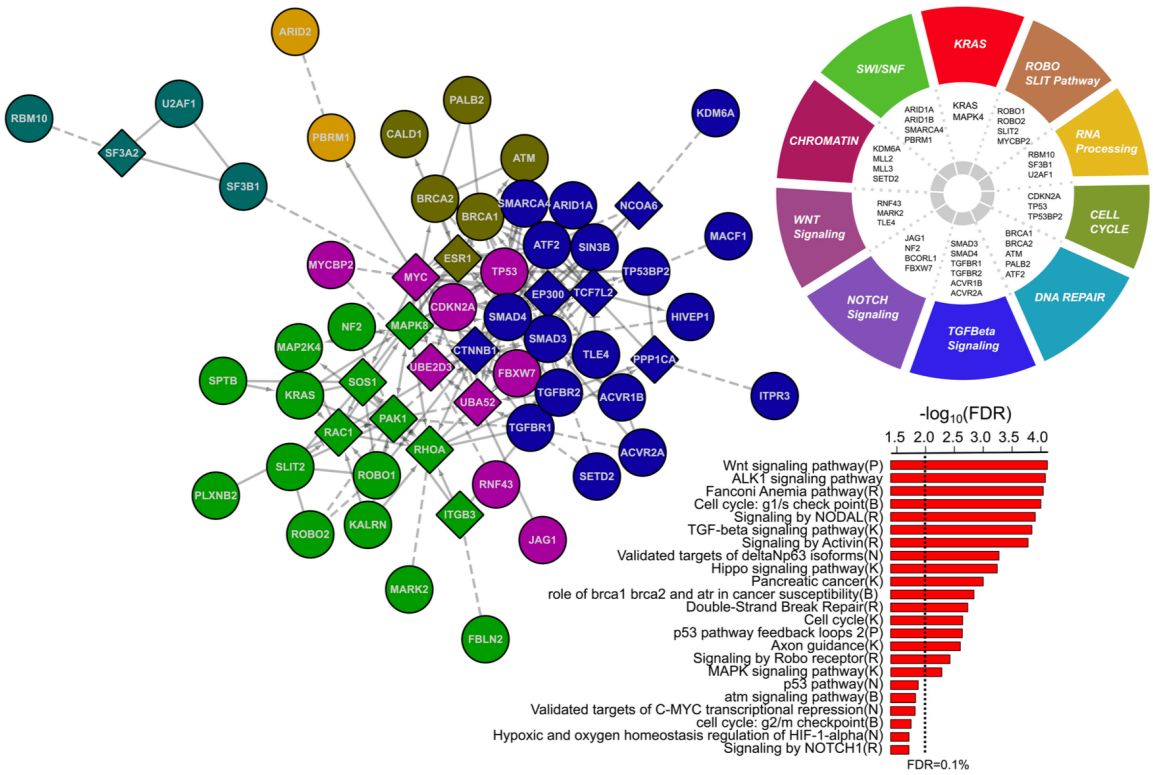


Figure 2. Pancreatic cancer mutation functional interaction (FI) sub-network identified by the Reactome FI cytoscape plugin. Mutated genes are indicated as coloured circles and linker genes (that is, genes not significantly mutated but highly connected to mutated genes in the network) indicated as coloured diamonds. Different node colours indicate different network clusters or closely interconnected genes. P values represent FDR < 0.05. Pathways significantly enriched in the identified FI sub-network are shown in the accompanying bar graph. Linker genes were not included in the enrichment analysis. Pie chart representing significantly altered genes and pathways (Bailey *et al.*, 2016).

1.2 Clinical features, diagnostic methods and treatments

1.2.1 Clinical features

Pancreatic adenocarcinoma has a very poor prognosis compared to PanNET as it is typically diagnosed at a late stage (III or IV). This is usually due to the lack of identifiable symptoms at the early stage of the disease, and to the diverse symptoms of more advance grades depending of the exact tumor location (De La Cruz *et al.*, 2014). More than two-thirds of PDAC locates in the head of the pancreas presenting symptoms like abdominal pain, jaundice, dark urine, and acholic stools as a sign of biliary obstruction

(American Gastroenterological Association, 1999). Other non-specific symptoms could be unexplained weight loss, dyspepsia and fatigue. Similarly, PDAC patients may have normal physical examination in the early stages of their disease. Therefore, many investigation have been conducted to find specific and sensitive diagnostic tools to eliminate the delayed or missed diagnosis to the greatest extent.

1.2.2 Diagnostic methods

Abdominal ultrasonography is considered to be an easy imaging test for patients with common symptoms. Unfortunately, it is neither efficient nor sensitive as a diagnostic method for PDAC. The current and common diagnostic methods of suspected pancreatic cancer are based on computed tomography (CT), magnetic resonance (MR) as well as magnetic resonance cholangiopancreatography (MRCP) and endoscopic ultrasound (EUS) (Chu *et al.*, 2017). Besides those imaging technics, positron emission tomography (PET)/CT, endoscopic retrograde cholangiopancreatography (ERCP) and fine-needle aspiration are promising approaches to identify smaller tumors or isoattenuating tumors which may be undetectable by CT or MR.

Serum biomarkers have also been proposed for pancreatic cancer detection. Among them, carbohydrate antigen 19-9 (CA 19-9) is the most commonly used biomarkers for detecting PDAC. However, CA 19-9 has a limited sensitivity of 79% to 81% and specificity of 82% to 90% that restrict its application as a stand-alone test for diagnosis (Ballehaninna and Chamberlain, 2012). Currently, novel serum biomarkers such as DNA methylation, cell free nucleosomes, microRNAs and cell free DNA are being evaluated to improve detection and prediction (Pedersen *et al.*, 2011; Dauksa *et al.*, 2012; Bauden *et al.*, 2015; Komatsu *et al.*, 2015; Kinugasa *et al.*, 2015). Although the combination of these serum biomarkers or together with imaging technology can improve the detection of PDAC, they are still not applicable for early detection as either neoplastic cells or the cells in the microenviroment evolve in their protein expression during the progression of pancreatic cancer (Nolen *et al.*, 2014).

1.2.3 Treatments

Treatments for PDAC include surgical resection, chemotherapy, radiation, chemoradiotherapy and immunotherapy (Tempero *et al.*, 2017; Aroldi and Zaniboni, 2017). With the help of multidisciplinary consultation, tumors are classified as resectable, borderline resectable, or unresectable (locally advanced and metastatic) (Li *et al.*, 2004). Whatever management chosen aims to palliate symptoms and improve survival time, as well as takes patient tolerance into consideration.

- Resectable and borderline resectable tumors

There is no doubt that surgical resection is the only potentially curative treatment for PDAC patients. However, less than 20% of patients have the opportunity to undergo surgery and the median survival time after adjuvant therapy is less than 2.5 years (Tempero *et al.*, 2017). Carcinoma located in the head of pancreas has more chance to be resected than located in the body or tail of the pancreas. The classic surgery for resecting the tumor in the head of pancreas is called Whipple procedure. Besides, postoperative (Adjuvant) therapy, preoperative (Neoadjuvant) therapy and even adjuvant treatment after neoadjuvant therapy have been used after surgical resection.

- Locally advanced tumors and metastasis

The National Comprehensive Cancer Network (NCCN) recommends systemic chemotherapy followed by consolidation chemoradiation therapy as a treatment option for patients with locally advanced tumors or metastasis. For example, combination chemoradiation therapy with FOLFIRINOX (flurouracil/leucovorin/oxaliplatin/irinoecan) (Gourgou-Bourgade *et al.*, 2013; Conroy *et al.*, 2011). Two common techniques of radiation therapy called intensity-modulated radiotherapy and stereotactic body radiotherapy (SBRT) also have been applied for clinical use (Tempero *et al.*, 2017). However, their benefits are quite limited and the treatment of advanced disease still remains controversial.

- Palliative care

For patients with symptoms such as biliary obstruction, gastric outlet obstruction and malnutrition, palliative care is of great importance. Endoscopic biliary stent, enteral stent or laparoscopic gastrojejunostomy and oral pancreatic enzyme replacement and parenteral nutrition supplying can be better options for these patients who lose the chance for surgical resection, chemotherapy or radiation (Moss *et al.*, 2006; Jeurnink *et al.*, 2007; Nagaraja *et al.*, 2014; Sikkens *et al.*, 2010)

- Immunotherapy

The immune system has been hypothesized to play a key role in pancreatic cancer due to the unique features in tumor microenvironment (dense stroma and immunosuppressive mediators). Therapies acting on innate or adaptive immunity are being investigated. The majority of immunotherapy clinical trials conducted includes immune checkpoint inhibitors (such as CTLA4, CD40), allogeneic (GM-CSF), autologous (MUC1) and vaccines (such as Gastrin-17, Hedgehog, GVAX). Although some interesting results point immunotherapy is a promising field, there are still many unknowns to be discovered (Aroldi and Zaniboni, 2017).

1.3 The origin and initiation of pancreatic cancer

Based on the above summarized clinical characteristics, it is easy to understand why most of PDAC-patients are diagnosed with advanced and incurable stages of tumors that favor a high mortality rate. Lack of current efficient therapeutic options is not helping and highlight the need to better comprehend the course of the disease from the earliest stages. Over the last decades, three common precursor lesions have been described for PDAC. They include pancreatic intraepithelial neoplasias (PanINs), intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs). Since those lesions consist of cells that resemble ductal cells, it has been long assumed that ductal cells are the cell origin of PDAC. Recent studies from genetically engineered mouse models (GEMMs) have facilitated the study of PDAC-origin pointing acinar cells as one of the major and plausible source of pancreatic cancer.

In this section, I will cover the histology of precursor lesions and their genetic origin. Subsequently, I will summarize the current knowledge about the suspected cellular origin involved in the initiation of PDAC.

1.3.1 PDAC-precursor lesions

- Pancreatic Intraepithelial Neoplasia (PanIN)

Pancreatic intraepithelial neoplasias (PanINs) are the most common and important precursor lesions of PDAC. They arise in the form of small intralobular pancreatic ducts, with microscopic flat or papillary lesions measuring less than 5mm (Hruban *et al.*, 2004). PanINs are composed of columnar to cuboidal cells and they are classified morphologically into three grades: PanIN-1(including PanIN-1A and PanIN-1B), PanIN-2 and PanIN-3 [Figure 3]. PanIN-1 lesions are low-grade lesions which can be flat (PanIN-1A) or papillary (PanIN-1B). They are composed of columnar epithelial cells with round nuclei with basal cellular location (Hruban *et al.*, 2001). PanIN-2 lesions are intermediate-grades with presenting nuclear changes such as crowding, pleomorphism, hyperchromasia, pseudostratification and loss of nuclear polarity. Yet they are architecturally slightly more complex than PanIN-1 (Hruban *et al.*, 2001). PanIN-3 lesions show severe cytological and architectural atypia. They are forming papillae and cribriform structures, and they present clusters of cells arising from the epithelium that are stretching into the lumen of the duct. The nuclei in this "carcinoma in situ" grade are enlarged, pleomorphic, and poorly oriented (Hruban *et al.*, 2001).

Mucin markers MUC1, MUC2 and the intestinal differentiation marker CDX-2 are commonly used for identification or distinction of PanIN lesions. MUC1 is almost uniquely expressed in PanIN-2, PanIN-3 and invasive PDAC (Maitra *et al.*, 2003; Nagata *et al.*, 2007). In contrast to MUC1, MUC2 and CDX-2 are used to distinguish lesions type due to their sole expression in IPMNs and absence in PanINs and PDAC (Distler *et al.*, 2013; Adsay *et al.*, 2002; Lüttges *et al.*, 2001) [Table 1].

- Intraductal Papillary Mucinous Neoplasm (IPMN)

Pancreatic intraductal papillary mucinous neoplasms (IPMNs) are the most common cystic tumors of the pancreas. They have been proved to be the precursor of invasive PDAC. IPMNs are larger than 1cm compared to PanINs (Hruban *et al.*, 2004). Longer and more mucinous papillae lead to cystic dilatation of involved ducts which are more easily seen by gross anatomy and by imaging technology (Brugge, 2005). According to the site of origin, IPMNs are subdivided into main-duct (MD-IPMN) and branched-duct (BD-IPMN) sub-groups. MD-IPMNs include three subtypes called intestinal, pancreatobiliary, and oncocytic, while BD-IPMNs are refered to the gastric subtype (Tanaka *et al.*, 2012) [Figure 4]. Based on the degree of dysplasia, IPMNs are classified into low-grade dysplasia, intermediate-grade dysplasia, high-grade dysplasia, and IPMN with associated invasive carcinoma (Bosman *et al.*, 2010).

Intestinal IPMNs consist of tall columnar cells with elongated nulei and amphophilic cytoplasm. These lesions frequently present intermediate-grade or high-grade dysplasia (Zamboni *et al.*, 2013). They express MUC2, MUC5, CDX-2, and MUC4 (Basturk *et al.*, 2010; Kitazono *et al.*, 2013). The pancreatobiliary IPMN is a rare subtype, characterized by complex branched papillae with high-grade dysplasia. Those lesions are positive for MUC1 and MUC5 assessed by immunohistochemical staining (Distler *et al.*, 2014). The oncocytic IPMN is another rare subtype showing high-grade dysplasia associated with invasive capacity. The lining cells in the complex arborizing papillae reveal eosinophilic cytoplasm mixed with goblet cells. It has a diffuse and inconstistent expression of MUC1, MUC2, MUC5, and MUC6 (Basturk *et al.*, 2010; Distler *et al.*, 2014). The gastric subtype is mainly found in BD-IPMNs with epithelial cells resembling gastric foveolar cells. Most of these lesions are low-grade dysplasia with a consistent expression of MUC5 and MUC6 (Distler *et al.*, 2014; Basturk *et al.*, 2010) [Table 1].

- Mucinous Cystic Neoplasm (MCN)

Compared to PanINs and IPMNs, mucinous cystic neoplasms (MCNs) are the least frequent precursor lesions of PDAC. The cystic lesions in MCNs can grow into large structures presenting seperated cysts and intra-cystic calcifications. MCNs lesions consist of columnar epithelial cells with different levels of dysplasia [Figure 5]. MCNs are classified into invasive and non-invasive MCNs. The latter can be further classified into low-grade dysplasia, intermediate dysplasia, and high-grade dysplasia.

For identification of MCNs, several markers are used by immunochemical staining. The epithelial cells are positive for the mucin markers MUC5, MUC2, and MUC1 (expressed in high-grade or invasive lesions) and for the epithelial markers cytokeratin7, and 19 [Table 1]. Besides, the progesterone receptor, estrogen receptor, vimentin, and smooth-muscle actin are also reported to be detectable for the presence of an ovarian-like stroma in MCNs (Zamboni *et al.*, 1999; Zamboni *et al.*, 2013).

Type of precursor lesions	Expression profile	Lesion grades
PanINs		
PanIN-1	MUC1(-), MUC2(-), CDX-2(-)	low-grade
PanIN-2 and 3	MUC1(+), MUC2(-), CDX-2(-)	intermediate and high-grade
IPMNs		
Intestinal IPMNs (MD-IPMN)	MUC2(+), MUC4(+), MUC5(+), CDX-2(+)	intermediate- and high-grade
Pancreatobiliary IPMNs (MD-IPMN)	MUC1(+), MUC5(+)	high-grade
Oncocytic IPMNs (MD-IPMN)	MUC1(+), MUC2(+), MUC5(+), MUC6(+)	high-grade (associated with invasive carcinoma)
Gastric IPMNs (BD-IPMN)	MUC5(+), MUC6(+)	low-grade
MCNs	MUC1(+), MUC2(+), MUC5(+)	high-grade or invasive lesions

Table 1. Specific markers in different types of precursor lesions.

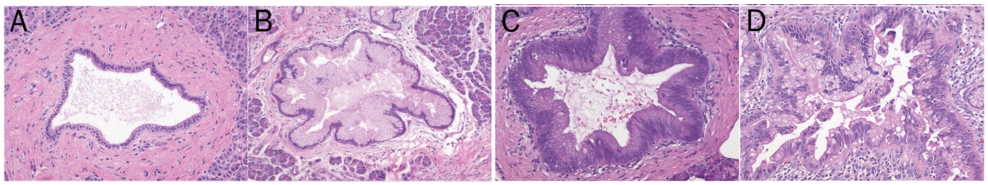


Figure 3. The progression from normal ducts (A) to PanIN (B-D) is associated with both architectural and cytological changes. In PanIN-1 the nuclei are uniform and basally oriented (B). The nuclei in PanIN-2 are slightly larger, more basophilic and there is some loss of nuclear polarity (C). The nuclear pleomorphism in PanIN-3 can be significant (D) (Hruban *et al.*, 2008).

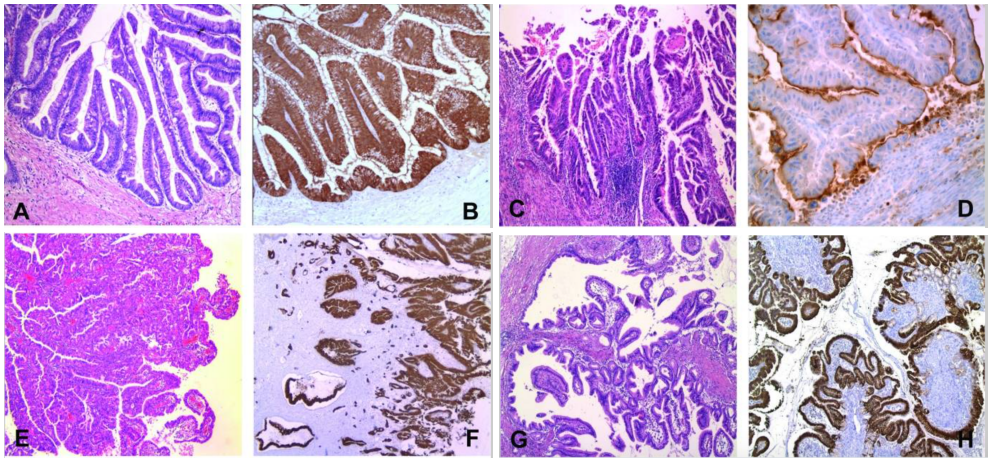
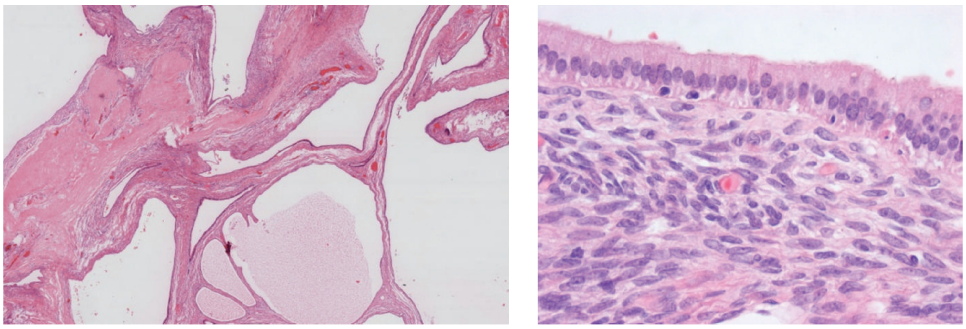


Figure 4. Histopathological subtypes of IPMN of the pancreas and their typical mucin patterns. (A, B): IPMN of the intestinal type positive for MUC2. (C, D): IPMN of the pancreatobiliary type positive for MUC1. (E, F): IPMN of the oncocytic type showing positivity for MUC5. (G, H): IPMN of the gastric type, positive for MUC5 (Grützmann *et al.*, 2010).



(a) (b)

Figure 5. The histology of MCN. (a) Overview of a multicystic MCN (H&E 2x) with cuboidal to columnar epithelial lining, mild dysplasia, and underlying ovarian type stroma. (b) High power view of MCN with columnar epithelial lining and underlying ovarian type stroma (H&E 40x) (Distler *et al.*, 2014).

1.3.2 Mutation and genetics of PDAC

The "big four" mutated genes responsible of the onset of PDAC include V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*), cyclin-dependent kinase inhibitor p16 (*p16*)/cyclin-dependent kinase inhibitor 2A (*CDKN2A*), tumor protein 53(*Tp53*), and SMAD family member 4 (*SMAD4/DPC4*) together with telomerase shortening are involved in the progression (Hruban *et al.*, 2004).

Telomerase shortening and *KRAS* oncogenetic mutations appear first in low-grade PanINs lesions, followed by the inactivation of *p16/CDKN2A* in intermediate-grade lesions, and finally the inactivation of *Tp53* and *SMAD4/DPC4* in high-grade PanINs (Koorstra *et al.*, 2008; Moskaluk *et al.*, 1997; Lüttges *et al.*, 2001). Besides, a stepwise increase of *KRAS* and *p16/CDKN2A* in the prevalence of mutated genes has been found with the degree of dysplasia of PanINs (Löhr *et al.*, 2005; Wilentz *et al.*, 1998).

Although some of IPMNs have the same genetic alterations as found in PanINs such as *KRAS*, *p16/CDKN2A*, *Tp53*, and *SMAD4* gene-mutations that are also found in the PDAC, specific mutations found in IPMNs have been reported (Fritz *et al.*, 2009). For example, meta-analysis performed by Lee *et al.* supports that both *KRAS* and *GNAS* mutations can be diagnostic markers for IPMN. Although these mutations are found in all subtypes, *GNAS* mutations are more frequently seen in the intestinal subtype and *KRAS* mutations are more frequently seen in the pancreatobiliary subtype (Lee *et al.*, 2016). Qiu *et al.* demonstrated that combined mutations affected the *ACVR1B* gene coding for the Activin Receptor type 1B and *KRAS* accelerate the growth of IPMNs in mice. These animals develop IPMNs composed of papillary, noninvasive epithelial lesions with mostly low-grade dysplasia (Qiu *et al.*, 2016). A small amount of IPMNs shows other mutations such as phosphatidylinositol-4,5-bisphosphate 3-kinase (*PIK3CA*) and v-Raf murine sarcoma viral oncogene homolog B1 gene (*BRAF*) (Schönleben *et al.*, 2007; Mohri *et al.*, 2012). Although the mutations mentioned above are helpful in identifying patients with IPMNs, their presence is limited in the early stage of IPMNs. For those individuals with high-grade dysplasia or invasive IPMNs, methylation and hence silencing of several genes (*BNIP3, PTCHD2, SOX17, NXPH1, EBF3*) have also been reported (Hong *et al.*, 2012).

The genetic changes in the formation and development of MCN remains unclear. *KRAS* mutations can be detected from the low-grade dysplasia to the advanced MCNs with an increasing frequency, while mutations of *p16, Tp53,* and *SMAD4* are found in

high-grade and invasive MCNs (Jimenez *et al.*, 1999; Yonezawa *et al.*, 2008). Whole-exome sequencing analysis showed that no mutations of *GNAS* occurred in MCNs which can be used as a distinguishing marker between IPMNs and MCNs (Wu *et al.*, 2011).

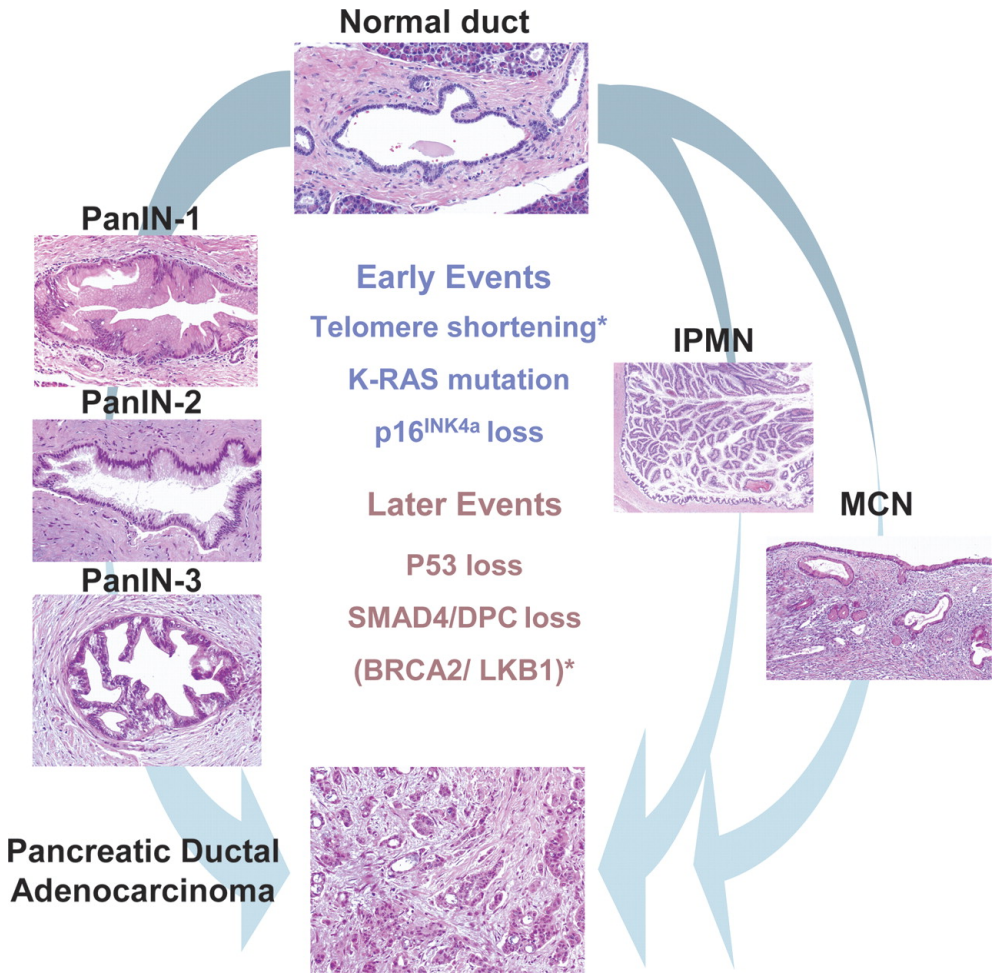


Figure 6. Pancreatic precursor lesions and genetic events involved in PDAC progression (Hezel *et al.*, 2006).

Taken together, from the histology of the three precursor lesions, it seems reasonable to think that pancreatic cancer arises from the normal ducts. Although they share common alteration of the "four major PDAC" genes mentioned above, the timing of their mutation is different during the initiation and development of precursor lesions, Kras mutation appearing to represent the main initiating drive-gene [Figure 6]. Moreover, from recent studies, some additional mutated genes were also reported to present in certain subtype or

grade in IPMNs (Lee *et al.*, 2016; Qiu *et al.*, 2016; Schönleben *et al.*, 2007; Mohri *et al.*, 2012; Hong *et al.*, 2012).

1.3.3 Mouse models of pancreatic neoplasms and Cell origin of PDAC

GEMMs have given the most solid evidence to illustrate the source of pancreatic neoplasms (Guerra C and Barbacid, 2013). The common GEMMs are based on Kras mutated mice to investigate pancreatic carcinogenesis. The genetically engineered Kras mice [Lox-stop-Lox (LSL)-Kras^{G12D}; LSL-Kras^{G12D}] were bred using mice expressing Cre recombinase under the control of a crucial pancreatic progenitor gene promoter [Pancreatic And Duodenal Homeobox 1 (*Pdx1*)-Cre or Pancreas Associated Transcription Factor 1a (*Ptf1a/p48*)-Cre]. Cre recombinase will eliminate a floxed stop codon and activate oncogenic Kras mutation, specially in the pancreas.

Pdx1 and *Ptf1a/p48* have been identified as key transcription factors in the development of pancreas (Kim and MacDonald, 2002; Ashizawa *et al.*, 2004). The first identifiable pancreatic progenitor cell in the pancreas arises in the dorsal and ventral endoderm at embryonic day 8 (e8) in fetal mouse. *Pdx1* is first detected at e8.5 and *Ptf1a/p48* is expressed slightly later at e9.5 (Ashizawa *et al.*, 2004; Obata *et al.*, 2001). During organogenesis, *Pdx1* is broadly expressed in all cells differentiating toward the exocrine and endocrine components of the islets of Langerhans. In the adult mouse, *Pdx1* expression is confined to the islet cells, but is almost undetectable in other pancreatic cell types (Park *et al.*, 2011; Ashizawa *et al.*, 2004). On the other hand, *Ptf1a/p48* is also necessary after the onset of pancreatic bud formation. It was initially proposed to be required principally for the wave of acinar cell differentiation that begins around e13.5 and to commit pancreatic precursor cells to the acinar fate (Krapp *et al.*, 1996; Krapp *et al.*, 1998). In the adult pancreas, *Ptf1a/p48* expression is restricted to acinar cells.

To make you better understand the following part, I will first try to show you some GEMMs with prenatal Cre-mediated recombination targeting all pancreatic epithelium [Table 2]. As Kras mutation alone are not sufficient to lead the development of PDAC, additional gene mutations are needed for further progression to adenocarcinoma, consistent with the multiple genetic alterations in human PDAC (Hingorani *et al.*, 2003; Bailey *et al.*, 2016). The deletions or mutations of additional genes have been revealed to either

accelerate Kras-induced pancreatic tumorigenesis in GEMMs or cause other types of premalignant lesions such as IPMNs and MCNs [Table 2].

GEMMs	Promotor	Premalignant lesions	Reference
Pdx1-Cre; LSL-Kras^{G12D}	Pdx1	PanINs	Hingorani *et al.*
p48-Cre; LSL-Kras^{G12D}	Ptf1a	PanINs	Hingorani *et al.*
Pdx1-Cre; LSL-Kras^{G12D}; LSL-Trp53^{R172H}	Pdx1	Accelerated PanINs	Hingorani *et al.*
Pdx1-Cre; LSL-Kras^{G12D}; p53$^{lox/lox}$	Pdx1	Accelerated PanINs	Bardeesy *et al.*
Pdx1-Cre; LSL-Kras^{G12D}; p16$^{-/-}$	Pdx1	Accelerated PanINs	Bardeesy *et al.*
Pdx1-Cre; LSL-Kras^{G12D}; Ink4a/Alf$^{lox/lox}$	Pdx1	Accelerated PanINs	Aguirre *et al.*
Pdx1-Cre; LSL-Kras^{G12D}; Pten$^{+/lox}$	Pdx1	Accelerated PanINs	Kennedy *et al.*
Ptf1a-Cre; LSL-Kras^{G12D}; Tgfbr2$^{lox/lox}$	Ptf1a	Accelerated PanINs	Ijichi *et al.*
Pdx1-Cre; LSL-Kras^{G12D}; Brca2$^{-/lox}$	Pdx1	Accelerated PanINs	Skoulidis *et al.*
Pdx1-Cre; LSL-Kras^{G12D}; Notch1$^{lox/lox}$	Pdx1	High grade PanINs	Hanlon *et al.*
Pdx1-Cre; LSL-Kras^{G12D}; Lkb1$^{lox/lox}$	Pdx1	Accelerated PanINs	Morton *et al.*
Pdx1-Cre; LSL-Kras^{G12D}; Usp9x$^{+/lox}$	Pdx1	Accelerated PanINs	Perez-Mancera *et al.*
p48-Cre; LSL-Kras^{G12D}; Atm$^{lox/lox}$	Ptf1a	Accelerated PanINs	Russell *et al.*
Pdx1-Cre; LSL-Kras^{G12D}; SST2$^{-/-}$	Pdx1	Accelerated PanINs	Chalabi-Dchar *et al.*
Pdx1-Cre; LSL-Kras^{G12D}; Smad4$^{lox/lox}$	Pdx1	IPMN	Bardeesy *et al.*
Pdx1-Cre; LSL-Kras^{G12D}; Acvr1b$^{lox/lox}$	Pdx1	IPMN	Qiu *et al.*
Ptf1a-Cre; LSL-Kras^{G12D}; Brg1$^{f/f}$	Ptf1a	IPMN	Figura *et al.*
Pdx1-Cre; LSL-Kras^{G12D}; Tif1γ$^{lox/lox}$	Pdx1	IPMN	Vincent *et al.*
p48-Cre; LSL-Kras^{G12D}; Smad4$^{lox/lox}$	Pdx1	MCN	Izeradjene *et al.*
Ptf1a-Cre; LSL-Kras^{G12D}; Notch2$^{lox/lox}$	Ptf1a	MCN	Mazur *et al.*

Table 2. GEMMs with prenatal Cre-mediated Recombination (Modified from Yamaguchi *et al.*, 2018).

We know that the exocrine pancreas is the region where the most common type of pancreatic cancer arises. However, it still remains unclear how normal exocrine cells (i.e. **acinar cells** and **ductal cells**) become pre-cancerous and then cancerous. In recent years, some reports the implication of **centroacinar cells** have also been pointed as a cell population from which the pancreatic tumor could initiate [Figure 7].

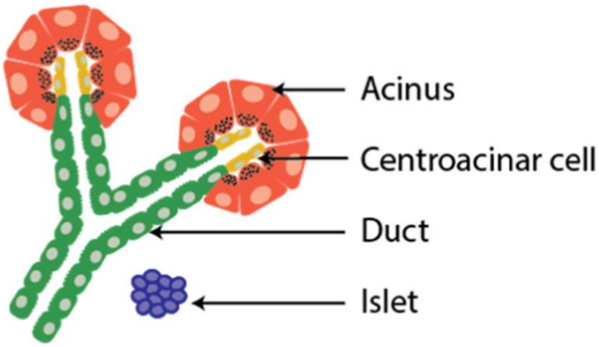


Figure 7. Functional units of the adult pancreas are made up of acinar, centroacinar, and ductal cells and are interspersed with islets (Iismaa *et al.*, 2018).

Because *Pdx1* and *Ptf1a/p48* are transcription factors with pan-epithelial expression, the genetic approach in KC mice (*Pdx1-Cre/LSL-Kras*^{G12D} *or Ptf1a-Cre/LSL-Kras*^{G12D}) leads the targeting of mutated Kras to the whole pancreas and does not allow us to identify the specific cells responsible for the origin of PanINs and PDAC. Furthermore, these KC mice induce "prenatal", Cre-mediated recombination, and genetic alteration, which is unlikely in natural development and carcinogenesis . Conversely, in genetically engineered mice with inducible Cre, Kras is activated in specific types of adult cells to see if "matured" pancreatic cells can initiate pancreatic carcinogenesis. These techniques allow us to find the cells of origin, not only for pancreatic adenocarcinoma, but also for their precursor lesions.

Here, I will give some examples to summarize three cell origin hypothesis as potential sources of precursor and cancerous lesions mainly based on GEMMs with inducible Cre [Table 3].

Promotor	Targeted cell type	Cells of origin	References
Hnf1b	Ductal and centroacinar cells	IPMNs from duct cells	von Figura *et al.*
Hnf1b	Ductal and centroacinar cells	PDAC from duct cells	Bailey *et al.*
CK19	Ductal cells	PanINs from duct cells	Ray *et al.*
Elastase	Acinar cells	PanINs from acinar cells	De La *et al.*
Elastase	Acinar cells	PanINs from acinar cells	Habbe *et al.*
Mist1	Acinar cells	PanINs from acinar cells	Shi *et al.*
Ptf1a Sox9	Acinar cells Ductal and centroacinar cells	PanINs from acinar cells not from ductal cells	Kopp *et al.*
Elastase	Acinar cells	PDAC from acinar cells	Ji *et al.*
Elastase	Acinar and centroacinar cells	PanINs from acinar or centroacinar cells	Guerra *et al.*
Pdx1	Likely centroacinar cells	PanINs and PDAC from centroacinar cells	Stanger *et al.*

Table 3. Representative studies indicating the cell origin of pancreatic neoplasms (Modified from Yamaguchi *et al.*, 2018).

- Ductal cells

Pancreatic cancer and the precursor lesions mentioned above consist of cells that resemble ductal cells. Therefore, since a long time ago, ductal cells are believed to be the cellular source of origin of PDAC. IPMNs as another common precursor for PDAC show distinctive intraductal growth, which may reflect their ductal origin. One mouse model from Von Figura *et al.* supported this observation (von Figura *et al.*, 2014). In this study, they found that the loss of a core subunit of SWI/SNF chromatin remodelling complexes called Brahma-related gene 1 (Brg1) promotes oncogenic Kras induced preneoplastic transformation in adult duct cells resulting in the formation of IPMNs. In 2016 Bailey *et al.*

combined a lineage-tracing approach and the use of cre mice allowing the tamoxifen inducible activation of Kras mutation in pancreatic duct-cell (Hnf1b:CreER^{T2}) or in pancreatic acinar cell (Mist1:CreER^{T2}) to demonstrate that ductal cells mutated for p53 serve as an effective cell of origin of PDAC (Bailey *et al.*, 2016). In addition, although it is more unusual, a ductal origin of PanINs is also possible. Ray *et al.* used cytokeratin 19 (CK19) as a promotor to generate a mouse model called CK19^{CreERT}; LSL-Kras^{G12D} mice, because CK19^{CreERT} recombines loxP-flanked alleles in ductal cells but rarely recombines in islet and acinar cells (with less than 1%) (Means *et al.*, 2008). They then demonstrated that large ducts are capable of giving rise to mucinous neoplasms characteristic of early PanIN leisons (Ray *et al.*, 2011).

- Acinar cells

Several studies using inducible Cre strains such as *Elastase*-Cre and *Mist1*-Cre support the acinar origin of PanINs lesions (De La O *et al.*, 2008; Habbe *et al.*, 2008; Shi *et al.*, 2009). Work using Ptf1a and Sox9 gene promotors to drive the respective Cre-mediated activation of Kras in acinar cells and centroacinar cells have be done to study the origin of PanINs (Kopp *et al.*, 2012). Such work revealed that PanINs predominantly arise from Kras^{G12D} expression in acinar but not centroacinar cells. Ji *et al.* used a *Ela*^{CreERT};CAG-lox-GFP-lox-Kras^{G12D} mouse model to demonstrate that higher Ras activity in elastase expressing acinar cells results in rapid formation of PanINs and PDAC (Ji *et al.*, 2009). Besides these observations, Guerra *et al.* reported that selective expression of Kras^{G12V} in cells of acinar or centroacinar lineage induces PanINs and invasive PDAC by using a Elas-tTA; tetO-Cre; LSL-KRAS^{G12V} mouse model (Guerra *et al.*, 2007). Together, all these observations strongly suggest that acinar cells can transdifferentiate to aquire a ductal identity and by this mean could stand at the origin of PanINs.

- Centroacinar cells

Although most literatures propose that acinar and/or duct cells are the cell origin of the precancerous/cancerous pancreatic lesions, it is important to note that some report suggest that centroacinar cells could also lead to the formation of PanINs and precancerous pancreatic lesions. Result from Guerra's study indicates that centroacinar cells are a

possible origin for PanINs and PDAC, another study using two mouse model called Pdx1-Cre; Pten$^{lox/lox}$ and Pdx1-Cre; Pten$^{lox/lox}$; p53$^{-/-}$ with pancreas-specific Pten deletion also suggested that centroacinar cells are more likely the origin for PanINs and PDAC (Stanger *et al.*, 2005)

Here, I listed the representative studies mentioned above indicating three cell types of origin of pancreatic neoplasms [Table 3]. However, it is also worth mentioning that a number of studies point endocrine, progenitor or pancreatic duct glands (PDG) can be the origin of precursor or cancerous pancreatic lesions (Gidekel Friedlander *et al.*, 2009; Westphalen *et al.*, 2016; Siveke *et al.*, 2007; Yamaguchi *et al.*, 2016).

1.4 Acinar-to-ductal metaplasia (ADM) a mechanism of PDAC initiation

1.4.1 Mechanism of ADM and acinar plasticity

More and more studies shed light on acinar cells to be the commited population during pancreas tumorigenesis. Moreover, acinar cells show the highest plasticity among all the adult cell lineages of the pancreas and are proposed as the predominant initiating cells for PanINs and PDAC as discussed above. Pancreatic acinar cell can transdifferentiate to a duct-like cell, this process is termed acinar-to-ductal metaplasia (ADM) [Figure 8]. A number of dedicated transcription factors and molecules have been show to play a key role during ADM [Table 4].

ADM is associated with a progressive loss of acinar markers (such as Ptf1a, Amy2, Rbpjl, Cela1 and Cpa1) and a progressive gain of duct markers (Pdx1, Sox9, Hes1, Rbpj, and Ck7). Such process is driven by a series of transcription factors that have been shown to be important for the development of the pancreas. Here, I will give you details about some key transcription factors to explain the molecular mechanism of ADM.

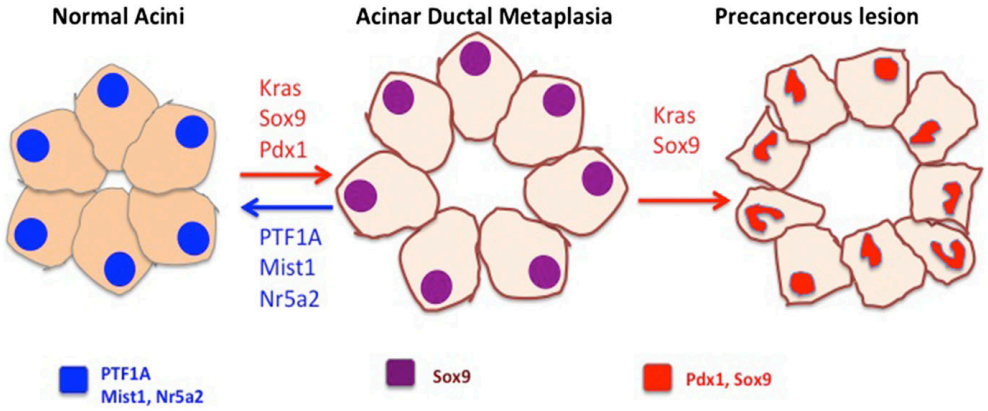


Figure 8. Acinar-to-ductal metaplasia arising from normal acini is an origin of pancreatic cancer (Schofield and Pasca di Magliano, 2015).

Acinar identity markers	Acinar transdifferentiation and ductal markers
Ptf1a or p48, Rbpjl, **Mist1**, GATA6, NR5A2, DICER1, PAF1, *et al.*	**Sox9**, **Pdx1**, Rbpj, **Hes1**, Myc, Klf4, *et al.*
Digestive enzymes such as AMY2, CPA1, ELA1, CELA1, CTRB1, *et al.* (Whitcomb and Lowe, 2007; Yasunaga *et al.*, 2018; Zhou *et al.*, 2007; Tamura *et al.*, 2018; Shi *et al.*, 2013)	Ck19, Ck7, Ck20, *et al.* (Bouwens, 1998)

Table 4. Acinar identity markers, acinar transdifferentiation and ductal markers. AMY2: alpha amylase; CELA1: chrymotrypsin-like elastase 1; CPA1: carboxypeptidase A1; CTRB1: chymotrypsinogen B1; ELA1: elastase 1.

- Ptf1a/p48

Over the last few years, *in vivo* work through the use of GEMMs have identified several transcription factors that are capable of promoting the transdifferetiation of acinar cells during ADM and the development of pancreatic cancer. Among them, the most important regulator appears to be the Pancreas Transcription Factor 1 complex (PTF1). The PTF1 complex take a role in the development of the exocrine pancreas, it is a trimeric transcription factor composed of a dimer of *PTF1a/p48* (a pancreas and neural restricted basic helix-loop-helix [bHLH] protein), a class A bHLH protein, and recombining binding protein suppressor of hairless (RBPJ) (Beres *et al.*, 2006). As acinar cell starts to develop, *Rbpjl* gradually replaces *Rbpj* (Masui *et al.*, 2010). The recruitment of p300/CREB by the

PTF1 complex then acetylates *Ptf1a* resulting in the increasing transcriptional activity, which can be inhibited by ICAT (an inhibitor of β-catenin and Tcf4) (Campos *et al.*, 2013). It has been established in Benitz's study that the *Ptf1a* gene is epigenetically silenced in ADM and in pancreatic tumor cells (Benitz *et al.*, 2016). Expression of *Ptf1a* is downregulated in both mouse and human PanINs. Besides those observations, it is reported that the sole loss of *Ptf1a* alone is sufficient to induce ADM, enhance inflammation, and sensitize pancreata to early KRAS-mediated PanIN initiation and rapid progression to PDAC (Krah *et al.*, 2015).

- Pdx1

Pdx1 and SRY-related HMG box factor 9 (Sox9) are key factors that regulate acinar cell transdifferentiation to a ductal phenotype. *Pdx1* is important for both the embryologic development of the pancreas and the maintenance of mature pancreatic islets (Ashizawa *et al.*, 2004). *Pdx1* is expressed at low levels in acinar cells in the adult mouse pancreas (Park *et al.*, 2011). *Pdx1* is upregulated in all types of precursor lesions, PDAC, pancreatic endocrine neoplasms and pancreatitis (Park *et al.*, 2011; Miyatsuka *et al.*, 2006; Song *et al.*, 1999). Upregulation of *Pdx1* in the adult pancreas leads to the formation of ADM induced by overexpression of transforming growth factor α (TGFα) (Song *et al.*, 1999). By crossing the CAG-CAT-PDX1 mice with Ptf1a-Cre mice, Miyatsuka *et al.* demonstrated that sustained expression of *Pdx1* is sufficient to induce ADM. Moreover, they revealed that activation of the Signal Transducer and Activator of Transcription 3 (Stat3) proetein is essential during the process of pancreatic metaplasia (Miyatsuka *et al.*, 2006).

- Sox9

Another indispensable transcription factor for transdifferentiation of acinar cells is Sox9. In normal mouse and human adult pancreas, Sox9 is absent from acinar and endocrine cells and only expressed in an average of 70% of pancreatic ductal cells, including terminal duct-cells centroacinar cells (Kopp *et al.*, 2011). Sox9 is ectopically expressed in pancreatic metaplastic acinar cells during ADM induced by inflammation or oncogenic Kras mutation (Prévot *et al.*, 2012; Kopp *et al.*, 2012). In recent years, one clinical study comparing Sox9 expression in different types of pancreatic neoplasms

showed that Sox9 is expressed in all stages of precursor lesions and PDAC. In detail, focal or diffuse Sox9 expression was detected in 100% PanINs, 100% IPMNs, 100% MCN and 89.0% PDAC (Shroff *et al.*, 2014). This high expression of Sox9 both in mice and in humans were proved to be correlated with the epidermal growth factor receptor (EGFR) pathway (Grimont *et al.*, 2015; Chen *et al.*, 2015; Hessmann *et al.*, 2016).

- Other transcription factors

In addition to the key transcription factors I mentioned above, here I will also give you some examples of other transcription factors during acinar transdifferentiaion. MIST1 (also known as BHLHA15) is also important for the normal development of exocrine pancreas and the acinar cell organization. Mist1 expression is restricted to acinar cells, where it serves to establish an apical-basal organization, position zymogen granules, and generate intercellular communication (Direnzo *et al.*, 2012). Loss of MIST1 accelerates the formation of ADM and Kras-induced PanINs with an increase of cell proliferation that was associated with a downregulation of p21(CIP1/WAF1) (Shi *et al.*, 2013; Shi *et al.*, 2009; Jia *et al.*, 2008). Expression of Hes1 leads to the transition from acinar cells to ADM and continues during the formation of precursor lesions and PDAC (Hingorani *et al.*, 2003). Other transcription factors such as GATA6, NR5A2, DICER1 and PAF1, MYC and Krüppel-like factor 4 (KLF4) are also reported to be involved in acinar-to-ductal reprograming (Martinelli *et al.*, 2016; von Figura *et al.*, 2014; Wang *et al.*, 2014; Dey *et al.*, 2014; Grippo and Sandgren, 2012; Wei *et al.*, 2016).

1.4.2 ADM as a precursor lesion in the evolution of PDAC

- In mice

Data from animal models show that cells that undergo ADM in response to oncogenic Kras mutation are precursors for pancreatic intraepithelial neoplasia lesions, which can further progress to PDAC (Morris *et al.*, 2010; di Magliano *et al.*, 2013). Oncogenic Kras activates a plenty of signalling pathways. Among them, the major signalling targets for activated Kras during ADM include canonical RAF/MEK/ERK pathway, the phosphatidylinositol 3-kinase (PI3K)/AKT, and serine/threonine-protein kinase D1

(PRKD1). PI3K activates ERK1/2 and AKT driving transdifferentiation of acinar cells, ADM, PanIN, and the formation of PDAC (Baer *et al.*, 2014; Shi *et al.*, 2013; Elghazi *et al.*, 2009; Albury *et al.*, 2015). STAT3 and nuclear factor of activated T cells 1 and 4 (NFATC1 and NFATC4) are activated via PI3K or AKT signalling. PI3K also induces cytoskeletal reorganization by activating small GTPases such as RAC1 and RHOA, controlling the reprogramming of acinar cells and regulating cell morphology (Baer *et al.*, 2014; Wu *et al.*, 2014). Another key signalling pathway that drives oncogenic Kras-induced ADM and progression to PanIN in mice is regulated by PRKD1. It can be activated by mutant Kras and wild-type Kras downstream of EGFR, and then initiates Notch and NF-κB signalling (Liou *et al.*, 2016; Liou *et al.*, 2015; Maniati *et al.*, 2011). Therefore, these two important pathways further drive the formation of precancerous lesions.

However, which of these effector pathways of oncogenic Kras control cell fate decisions and PDAC formation remains elusive. Besides, The persistent microinflammation caused by oncogene Kras-induced ADM process is not sufficient to drive further progression to PDAC, and additional inflammatory insults and genetic alterations are required for acceleration of the oncogenic induced process. In recent years, some paper has mentioned that downstream activin signalling pathway might be involved in early stages of pancreatic tumorigenesis. Thus, the role of this signalling pathway in the initiation and evolution of PDAC will be discussed in detail in next chapter of my thesis.

Besides oncogenic Kras-induced ADM, pancreatic inflammation or pancreatitis is also implicated in mediating ADM. Acute and chronic pancreatitis are common gastrointestinal disorders. Acute pancreatitis are at high risk for developing chronic pancreatitis, while chronic pancreatitis is a progressive fibroinflammatory disease with constant and disabling abdominal pain (Nøjgaard *et al.*, 2011; Witt *et al.*, 2007). Although most chronic pancreatitis patients don't develop PDAC and most PDAC patients don't have a history of pancreatitis, chronic pancreatitis is a well-known risk factor for pancreatic cancer. This maybe due to the overlapping inflammatory responses both in chronic pancreatitis and in PDAC, where similarities can be found in the desmoplasia and inflammatory infiltrates (Zheng *et al.*, 2013). Animal model studies of acute and chronic pancreatitis have been created to investigate mechanisms of pathogenesis, test therapeutic methods, and explore the influence of inflammation on the development of PDAC. Acinar cell transdifferentiation driven by inflammation results in the formation of ADM after

pancreatitis with a loss of acinar identity markers and an upregulation of duct markers (Liou *et al.*, 2013). Such observation supports the know predisposition character of pancreatitis in the onset of PDAC. Like oncogenic Kras driving the formation of ADM through its downstream signalling, inflammation also induces ADM through several molecular pathways. Transforming growth factor beta 1 (TGF-β 1) is believed to be one of the most potent fibrogenic modulators by activating cells that promote fibrosis and affecting acinar cell regeneration (Gress *et al.*, 1994; Menke *et al.*, 1997). Besides, activation of JAK-STAT3 signalling, EGFR signalling, Notch and NF-κ B signalling have been reported to drive the formation of inflammation-induced ADM (Corcoran *et al.*, 2011; Liou *et al.*, 2013; Sawey *et al.*, 2007; Chen *et al.*, 2015; Hessmann *et al.*, 2016). Therefore, as inflammation induced ADM shares some common signalling with oncogenic Kras induced ADM, it is reasonable to think that ADM is bridging the predisposing character of Pancreatitis in PDAC.

- In human

Although ADM as an initiating event for the development of pancreatic cancer has been demonstrated in mice, evidence that ADM has a role in the development of human PDAC is still not solid. One study using human tissue from patients with a PDAC family history reveals atypical flat lesions originating in areas of ADM might indicate that in human PDAC development could possibly initiate from ADM (Aichler *et al.*, 2012).

2 TGFβ/Activin signalling pathway

Transforming growth factor beta (TGFβ) signalling plays major roles in many biological and pathological condition such as embryonic development, homeostasis, tissue repair and cancer. From a molecular and cellular persepctive, member of the TGFβ signalling pathway have shown to contribute to various extent to cell proliferation, cell differentiation, cell migration, cell adhesion and remodeling of the cellular microenvironment. Considering this wide range of actions, it seems unavoidable that disruption of the pathway would have disastrous consequences on human health and drive pathologies such as cancer (Gordon and Blobe, 2008).

To better understand its role in cancer, in this chapter, I will firstly introduce the pathways and their different effectors. Then I will try to illustrate the role of activin signalling pathway in cancer especially in pancreatic cancer. And in the final part, I will present you the implication of TGFβ/Activin signalling in ADM.

2.1 TGF β superfamily and its signalling pathway

TGFβ superfamily consists of 33 members that have mainly been discovered due to their different roles in development. This family is split into two on the basis of both protein homology and the downstream effectors activated. The TGFβ-like family is composed of TGFβ ligands, Activin and Nodal, while the other part of the superfamily includes the bone morphogenic proteins (BMPs) and most of the growth and differentiation factors (GDFs) ligands.

2.1.1 Members of the pathway

- The superfamily of TGFβ ligands

i. <u>TGFβs</u>: *TGFB1, TGFB2, and TGFB3* genes encode TGFβ1, TGFβ2, TGFβ3 ligands. TGFβ ligands are first secreted inactive and their mature forms result from the cleavage of a polypeptide chain called LAP (latency associated peptide) by proteases such as furins (Harrison *et al.*, 2011).

ii. <u>Activins and Inhibins</u>: *INHBA* (Inhibin βA), *INHBB* (Inhibin βB), *INHBC* (Inhibin βC) and *INHBE* (Inhibin βE) are 4 different genes encoding different Inhibin β subunits. The Activin ligands are activating. Activin A and B are homodimers (Inhibin βA:Inhibin βA and Inhibin βB:Inhibin βB) whereas Activin AB is a heterodimer (Inhibin βA:Inhibin βB). INHA gene codes the Inhibin α and is the major subunit of Inhibin ligands. The Inhibins including Inhibin A and B are inhibiting heterodimers (Inhibin α :Inhibin βA and Inhibin α :Inhibin βB) and considered as Activins antagonists (Mason *et al.*, 1996; Risbridger *et al.*, 2001) [Figure 9].

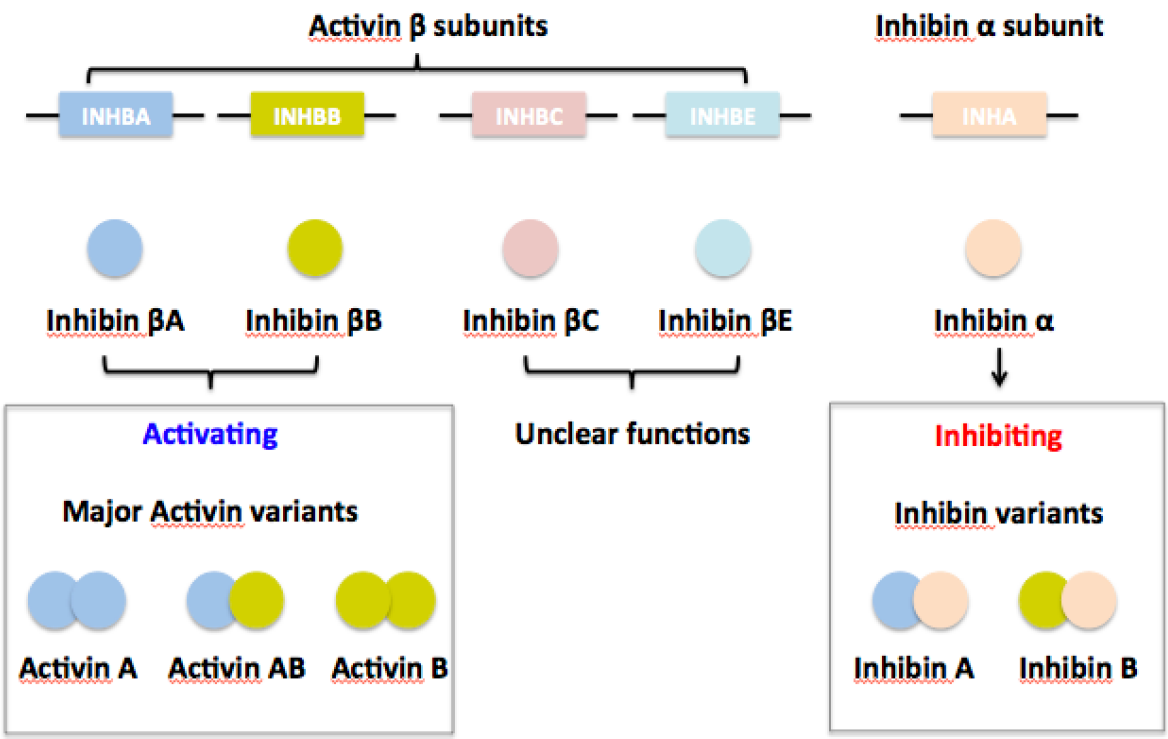


Figure 9. Activin and Inhibin ligands.

iii. <u>Other ligands in TGFβ/Activin group</u> include Nodal, Lefty1 and Lefty 2 encoded by Ndr, Lefty A/EBAF and Lefty B respectively (de Caestecher, 2004).

iiii. <u>BMP/GDF ligands</u> can be further divided into 9 subgroups: BMP2/4 (BMP2 and BMP4), BMP5/6/7 (BMP5, BMP6, BMP7, BMP8A and BMP8B), BMP9/10 (BMP9 and BMP10), BMP3 (BMP3 and BMP3b), GDF1 (GDF1 and GDF3), GDF5/6/7 (GDF5, GDF6 and GDF7), GDF9 (GDF9, GDF9b and GDF15), GDF8 (GDF8), GDF11 and MIS (de Caestecher, 2004).

- Receptors of the TGFβ superfamily

The functional complex required to sense TGFβ ligands is composed of two dimers of type I and type II transmembrane serine threonine kinase receptors. There are five type II receptors that have been identified in mammals, which display a constitutive kinase activity. Seven type I receptors were identified, all containing a glycine and serine rich domain (GS) essential for their activation via phosphorylation. Each ligand is specific of one pair of receptors. However, there are far more ligands than receptors, which means different ligands can recognize the same pair of receptors (Shi and Massagué, 2003). Here shows a summary of receptor-ligand interractions [Table 5].

	Receptor gene/protein	Ligands, binding partners	Receptor Smads
Type I receptors	*ACVRL1*/ALk1	ActivinA, BMP9, BMP10, TGF β 1, TGF β 3	1, 5, 8
	ACVR1/ALK2	ActivinA, ActivinB, BMP6, BMP7, MIS, TGF β 1, TGF β 2, TGF β 3	1, 5, 8
	BMPR1A/ALK3 (BMPRIa)	BMP2, BMP4, BMP6, BMP7, GDF6, GDF7	1, 5, 8
	ACVR1B/ALK4	ActivinA, ActivinB, GDF1, GDF3, GDF8, GDF11, Nodal	2, 3
	TGFBR1/ALK5 (T β RI)	TGF β 1, TGF β 2, TGF β 3, GDF8, GDF9	2, 3
	BMPR1B/ALK6 (BMPRIb)	BMP2, BMP4, BMP6, BMP7, BMP15, GDF5, GDF6, GDF9b, MIS	1, 5, 8
	ACVR1C/ALK7	ActivinB, ActivinAB, GDF3, Nodal	2, 3
Type II receptors	*ACVR2 (ACVR2A)*/ActRII	ActivinA, BMP2, BMP6, BMP7, BMP9, BMP10, GDF1, GDF5, GDF8, GDF9b, GDF11, InhibinA, InhibinB	
	ACVR2B/ActRIIb	ActivinA, BMP2, BMP6, BMP7, BMP9, BMP10, GDF5, GDF8, GDF11, InhibinA, InhibinB, Nodal	
	BMPR2/BMPRII	BMP2, BMP4, BMP6, BMP7, BMP9, BMP10, GDF5, GDF6, GDF9b	
	AMHR2/MISRII	MIS	
	TGFBR2/T β RII	TGF β 1, TGF β 2, TGF β 3	
Co-receptors	*TGFBR2*/ T β RIII	Inhibin, TGF β 1, TGF β 2, TGF β 3, BMP2, BMP4, BMP7, GDF5	
	ENG/Endoglin	In conjunction with appropriate type II receptor; ActivinA, BMP2, BMP7, TGF β 1, TGF β 3	
	CRIPTO/Cripto	GDF1, GDF3, Nodal, TGF β 1	
	RGMA/RGMa	BMP2, BMP4	
	RGMB/RGMb(DRAGON)	BMP2, BMP4	
	HJV/Hemojuvelin(RGMc)	BMP2, BMP4	
Pseudo-receptor	*BAMB*/BAMBI	Associates with Type I receptors	
Ligand antagonists	*CHRD*/Chordin	BMP2, BMP4	
	FST/Follistatin	ActivinA, ActivinB, Inhibin	
	LEFTY1/Lefty1	Binds to Cripto, competes with Nodal	
	NOG/Noggin	BMP2, BMP4, BMP7	
	SOST/Sclerostin	BMP2, BMP4, BMP6, BMP7	

Table 5. Receptor-ligand interactions in TGF β superfamily (Ouarné *et al.*, 2018; Loomans and Andl, 2016; Chen *et al.*, 2013; Gordon and Blobe, 2008; Bertolino *et al.*, 2008; Bernard *et al.*, 2006).

2.1.2 TGFβ/Activin signalling

- TGFβ/Activin canonical pathway through Smads

In general, TGFβ superfamily canonical pathway is initiated when ligand binds to a type II receptor. This binding further leads to recruitment, phosphorylation and activation of type I receptor followed by phosphorylation and activation of Smad proteins, which subsequently modulates the expression of different target genes in nucleus [Figure 10].

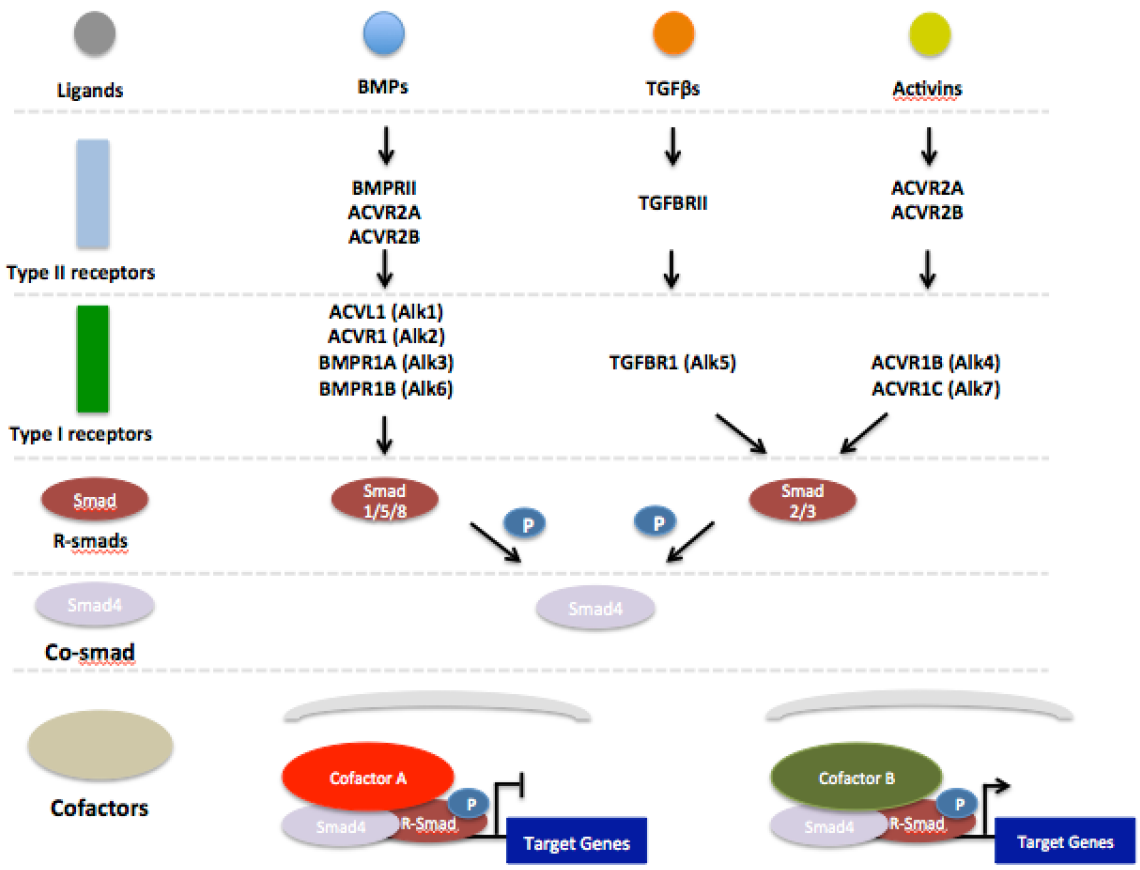


Figure 10. TGF β /Activin canonical pathway through Smads.

a. Smad mediators

Smad proteins mediate the intracellular activation of the pathway. Their structure consists of 2 main domains called MH1 and MH2 (for Mad Homology). The MH1 domain

allows DNA binding and contains a nuclear localization signal, while the MH2 domain is responsible for protein-protein interactions and transactivation (Ross and Hill, 2008; Zawel *et al.*, 1998). There are eight smads that can be subdivided in three different groups:

i. <u>The five receptor-activated smads (R-smad)</u> : **Smad2 and Smad3** are phosphorylated upon stimulation by **TGFβ/Activin ligands**, whereas **BMP-like ligands** drive the activation of **Smad1/5/8**. Smads are transcription factors that constantly shuttle between nucleus and cytoplasm with or without stimuli, which allows to continuously monitor receptors activation (Inman *et al.*, 2002; Xu *et al.*, 2002). They contain a carboxy-terminal SSXS domain phosphorylated by the activated type I receptors (Kretzschmar *et al.*, 1997). The specificity between R-smads and their receptors is controlled by a nine amino acid L45 loop found in the type I receptor and a L3 loop in their MH2 domain (Ross and Hill, 2008).

ii. The **<u>co-smad Smad4 (DPC4/MADH4)</u>** is a common modulator of the pathway shared between TGFβ/Activin and BMP signalling pathway (Massagué *et al.*, 2000). It forms a heterocomplex with R-smads, which translocates into the nucleus. Although it is a transcription factor required for most of the biological responses induced by TGFβ ligands, some can be independent of Smad4.

iii. The structurally divergent **Smad6 and Smad7** are inhibitory Smads (**<u>I-smad</u>**). They lack the MH1 domain promoting DNA interaction and thus, do not bind directly to DNA. I-smads expression is tightly regulated since they are targets of the TGFβ pathway and then represent a negative feedback loop for the activation of the pathways (Massagué, 2000).

b. TGFβ/Activin canonical pathway

Without any stimulation, receptors are present at the surface membrane as homodimers. Binding of the ligand brings together type I and type II receptors. Type II receptors phosphorylate and activate type I receptors in their GS domains, therefore changing their conformation and allowing recruitment and phosphorylation of Smad (Huse *et al.*, 2001). Adaptor proteins, such as Smad anchor for receptor activation (SARA), can facilitate the the interaction between type I receptor and R-smad (Tsukazaki *et al.*, 1998). Once released from the receptors, the R-smad form hetero-complexes with Smad4 and

translocate into the nucleus, where they modulate the expression of different target genes (Chacko *et al.*, 2004; Inman *et al.*, 2002; Xu *et al.*, 2002; Zhang *et al.*, 1996). In the nucleus, Smads are either ubiquitinated for degradation or continuously dephosphorylated. Both events drive to the dissociation of the complex, releasing inactive Smads followed by their export from the nucleus (Inman *et al.*, 2002; Xu *et al.*, 2002).

R-smad proteins bind to specific DNA sequences with low affinity. They need to synergize with additional transcription factors to efficiently and productively bind to DNA (Ross and Hill, 2008). Therefore, the Smad4-Rsmad-Cofactor allows DNA interaction to a combination of hundreds of target genes at once with high affinity. Each complex will target a specific DNA binding sequence and as a consequence will be responsible for the regulation of expression of a given set of genes. Furthermore, Smads can also interact with a number of co-activators, co-repressors or chromatin remodeling factors. Changes of these various players illustrate how and why a single stimulus can activate or repress genes depending on the cellular context (Ross and Hill, 2008). All these partners, which are for many tissue specific, are essential in determining which function the pathway will promote.

Although R-smad proteins show low affinity for DNA, they can bind directly to DNA after recognition of Smad Binding Element (SBE). Different SBE have been identified: Smad2/3 recognize CAGAC motifs, whereas Smad1/5/8 recognize GC rich sequences with GRCGNC motifs (Zawel *et al.*, 1998; Shi *et al.*, 1998). Multiple adjacent SBE are often located in DNA sequences, which may be important for cooperative effect to increase their DNA affinity and control gene expression (Liu *et al.*, 1997).

- TGFβ/Activin non canonical pathway

Smads are highly specific mediators of TGFβ/Activin pathway. However, series of reports indicate that TGFβ/Activin signalling is also involved in non-canonical pathways, in which their activation is independent of Smads. It can be processed directly through the receptors or with adaptor proteins as intermediates.

It has been described that TGFβ/Activin can activate the mitogen activated protein kinase (MAPK) pathways, including extracellular-signal-regulated kinase (ERK), p38 and Jun N-terminal kinase (JNK); PI3K/Akt pathway, and the NF-κB pathway (Derynck and

Zhang, 2003). TGFβ has also been reported as a modulator of small GTPase proteins such as Rho or Rac, which regulate cytoskeletal organization and gene expression (Ungefroren *et al.*, 2018). Besides, TGFβ has crosstalk with other signalling pathways such as WNT, Notch or Hippo (Xu *et al.*, 2018).

2.2 Activin and cancer

Activins and Inhibins were first described as gonadal proteins regulating synthesis and secretion of the follicle stimulating hormone (FSH). However, their expression is not restricted to reproductive tissues, since they have major roles in the embryonic development and in adults in processes such as bone growth, mesoderm induction, nerve cell survival, wound healing and tissue differentiation (Watabe and Miyazono, 2009).

The role of Activin-signalling in cancer have been far less studied compared to TGFβ. TGFβ-signalling has been known to have a dual role in cancer. In the premalignant state, TGFβ-signalling can either mediate cytostasis, differentiation or apoptosis mechanisms in a cell-autonomous manner or impact on stroma in a non-cell-autonomous manner to prevent the production of paracrine factors (Massagué, 2008). However, in the advanced stage of many cancers such as breast, glioma, prostate and melanoma, increased TGFβ-signalling has been reported to have a pro-tumoral effect (Levy and Hill, 2006). High levels of TGFβ ligands are often detected in the tumor environment, correlating with aggressiveness of cancers such as metastasis, epithelial-to-mensenchymal transition (EMT), immune system evasion, and angiogenesis (Massagué, 2008).

Similar to TGFβ, Activin-signalling has already been shown to impact processes such as cell growth, stemness, regulation of the microenvironment, cancer aggressiveness and cancer induced cachexia. Besides, the functions of activins via activin receptors are both cell type specific and contextual. In this part, I will try to summarize the previously described roles of Activin-signalling in different cancer.

- Cell growth inhibition and proliferation

Activins have a growth inhibition effect on breast, liver, prostate and pituitary adenoma cells. Study from neuroblastoma xenograft model, they revealed that using

ActivinA overexpressing human neuroblastoma cell lines results in an inhibition of neuroblastoma growth via the **Alk4/SMAD2-SMAD3 pathway** (Panopoulou *et al.*, 2005). In human breast cancer, ActivinA was reported to inhibit cancer cell proliferation and activates Smad2 and Smad3 resulting in cell cycle arrest with an upregulation of **cdk inhibitors p15, p21, and p27** (Burdette *et al.*, 2005). Another study from epithelial ovarian cancer demonstrated that activin inhibits cell proliferation by activation of canonical **Smad**, induction of **p15**, downregulation of proto-oncogenic CMYC levels and a G1 phase cell growth arrest (Ramachandran *et al.*, 2009). For the cancers originated from gastroenterological system, evidences supported that growth inhibition and apoptosis by activin signal is mainly through **SMAD-dependent** pathways (Hempen *et al.*, 2003; Kaneda *et al.*, 2011; Bauer *et al.*, 2012; Yokomuro *et al.*, 2000).

- Cancer aggressiveness

On the other hand, cancers require to escape the anti-tumoral function of activin signalling to became more advanced. This relies on the defects in several genes (such as smad, type I or type II receptor for activin) in different cancers and **activin paracrine role** in the tumor microenvironment. In early stage of prostate cancer, a decreased expression of *ACVR1B* suggests a loss of activin anti-tumoral function (van Schaik *et al.*, 2000). However, when prostate cancer becomes aggressive, tumor cells no longer respond to the growth inhibition of activin, although the epithelial cancer cells continue to secret activin. Instead, the elevated activin further increases angiogenesis and epithelial to mesenchymal (EMT) transition, and decreases immune surveillance favoring tumor progression (Gold *et al.*, 2012).

- Stemness

Another thing worth paying close attention is the self-renewal and differentiation of adult stem cells in promoting cancer progression. Since recent years, several reports suggested that Activin-signalling plays a key role in the self-renewal of adult stem cells. For example, Liu *et al.* demonstrated that colorectal cancer stem cell self-renewal is enhanced by activin pathway (Liu *et al.*, 2016). Moreover, its regulation in cancer stem

cells will further increase the plasticity and metastatic potential of tumor cells (Lonardo *et al.*, 2011).

- Cancer induced cachexia

Cancer cachexia is characterized by extreme skeletal muscle loss that results in high morbidity and mortality. The incidence of cachexia varies among tumor types. Elevated levels of circulating ActivinA have been noted in many cancer patients and are associated with severe cachexia and poor outcome (Fearon *et al.*, 2012; Han *et al.*, 2013; Loumaye *et al.*, 2015; Sartori *et al.*, 2014).

- Immune system

Activin-signalling has also been reported to play an important role on immune system during tumorigenesis. It has long been known to be a key modulator of immunity and inflammation. It regulates macrophage polarization, T lymphocytes differentiation, monocyte and dentritic ells migration and natural killer functions (Ogawa *et al*., 2011; Hedger *et al*., 2011; Sainz *et al*., 2015). However, the role of Activin-signalling in immune system is very complex and far from completely understood.

Although the pro-tumoral role of Activin-signalling seems to make it a potential target for therapies, the crosstalks with cell cycle regulation, cell fate decisions and adult tissue homeostasis suggest that targeting this pathway in cancer can be complicated and a more complete understanding of the mechanisms of Activin-signalling in cancer is therefore necessary to be well considered before.

2.3 Activin in PDAC

Over decades of years, numerous studies on the TGFβ signal in PDAC have been reported. Some provide evidence that the TGFβ signal functions as a tumor suppressor, while many others support that TGFβ/Activin Receptor-like kinase 5 (Alk5) mediated cell

migration or metastasis promote tumorigenesis in PDAC (Birnbaum *et al.*, 2012; Gaspar *et al.*, 2007; Zeeh *et al.*, 2016; Ungefroren *et al.*, 2017). Compare to the TGFβ signal, less studies regarding the Activin-signalling on PDAC have been reported.

In this part, I will first briefly introduce the implication of Activin-signalling in PDAC. Then I will try to summarize the representative literatures based on the viewpoints considering Activin-signalling as either a pro-tumoral role or anti-tumoral role, as well as in mediating cachexia in PDAC.

2.3.1 Implication of Activin-signalling in PDAC

Although most of the studies suggest that Activin-signalling promotes pancreatic cancer progression (for example, in modulating the role of cancer stem cells and pancreatic stellate cells), as well as mediates pancreatic cancer induced cachexia, a few papers emerged in recent years support that Activin-signalling acts as an anti-tumoral role in the progression of PDAC (Lonardo *et al.*, 2012; Parajuli *et al.*, 2018; Qiu *et al.*, 2016). Therefore, the role of the Activin-signalling in pancreatic carcinogenesis remains controversial.

Few studies have already confirmed the importance of the Activin-signalling pathway in pancreatic both through the use of *in vivo* and *in vitro* experimental models. Mutated genes within Activin-signalling have been reported to have an implication in PDAC more than 20 years ago (Hahn *et al.*, 1996; Kleeff *et al.*, 1998; Liu, 2001; Su *et al.*, 2001). In recent years, with the arising of the new classification in PDAC based on gene profiling and the analysis of Common Insertion Sites (CISs) in the recovered pancreatic neoplasms, the role of Activin-signalling has been further highlighted (Pérez-Mancera *et al.*, 2012; Bailey *et al.*, 2016; Cancer Genome Atlas Research Network, 2017).

SMAD4 is a common effector in the downstream of TGF β - and Activin-signalling (Massagué *et al.*, 2000). Inactivation of *SMAD4* coupled with activation of oncogenic *Kras* is one of the common events in pancreatic cancer, with a mutation frequency about 55% (Hahn *et al.*, 1996). However, evidences have showed that mutation of *SMAD4* is more frequently seen in higher grades of dysplasia in PDAC (Lüttges *et al.*, 2001; Fritz *et al.*, 2009; Yonezawa *et al.*, 2008; Hruban and Klimstra, 2014; Reid *et al.*, 2014;).

Early in 1998, Kleeff *et al.* first raised the important role of Activin-signalling in human pancreatic cancer (Kleeff *et al.*, 1998). In their work, they found all cell lines including ASPC-1, CAPAN-1, COLO-357, MIA-PaCa-2, PANC-1 and T3M4 expressed the activin β A subunit, whereas expression of β B and α subunits were absent. More interestingly, while *ACVR2* and *ACVR2B* were expressed in all cell lines, *ACVR1B* were only present in some pancreatic cancer cell lines (ASPC-1, CAPAN-1, COLO-357, and PANC-1). Further in situ hybridization showed that activin β A and *ACVR1B* were strongly expressed in both the diffuse infiltrative and duct-like cancer cells.

Since then, many researchers start to shed the light on this important Activin recetor---*ACVR1B*/ALK4. In 2001, Gloria's group first provide genetic evidence from human tumors to support *ACVR1B* as a tumor-suppressor gene (Su *et al.*, 2001). However, it still remains controversial since some others defended *ACVR1B* plays a pro-tumoral role in both autocrine and paracrine way in the late stage of PDAC (Lonardo *et al.*, 2011; Lonardo *et al.*, 2012). On the other hand, Gloria's group later demonstrated that a loss of *ACVR1B* favors an accelerated formation of IPMNs in Kras mutated mice, suggesting the presence of *ACVR1B* can impact the metaplastic changes in the pancreas (Qiu *et al.*, 2016). They further checked a potential differentiated marker called Dclk1 (doublecortin like kinase 1) in ADM, PanINs and IPMNs (Delgiorno *et al.*, 2014), which has been previously known as a marker of gastrointestinal tuft cells and stem cells (Gerbe *et al.*, 2011; Nakanishi *et al.*, 2013; May *et al.*, 2008). Their work demonstrated that Dclk1 labels a subpopulation in mice with *ACVR1B* ablation and Kras mutation that contribute to the initiation of IPMNs (Qiu *et al.*, 2018).

Besides *ACVR1B* mutation, some people identified point mutation of *ACVR2* (*ACVR2A)* is detected in human pancreatic cancer and supports it as a candidate tumor suppressor gene during tumorigenesis (Hempen PM *et al.*, 2003; Mann *et al.*, 2012).

2.3.2 Function of Activin-signalling in PDAC

- Pro-tumoral function of Activin-signalling

In 2011, study from Heeschen's group demonstrated that Activin and Nodal were hardly detectable in highly differentiated pancreatic cancer cells but notably overexpressed in Pancreatic Cancer Stem Cells (PCSCs) as well as in stroma-cancer-derived pancreatic

stellate cells (PSCs). Those authors demonstrate that knockdown or pharmacologic inhibition of ALK4 and ALK7 expression in PCSCs abrogated their **self-renewal capacity and tumorigenicity** *in vivo* (Lonardo *et al.*, 2011). They further show that such approach was reverting the resistance of orthotopically engrafted PCSCs to treatment with gemcitabine (Lonardo *et al.*, 2011). In a following publication Lonardo *et al.* reported that the secretion of Nodal and Activin by PSCs was promoting the stemness of PCSCs as assessed by sphere formation. They further demonstrate that PSCs were capable of promoting the invasiveness of PCSCs in an **ALK4-dependent** manner (Lonardo *et al.*, 2012). These data imply that Nodal/Activin contribute to the paracrine niche at the tumor-stroma interface, being capable of driving the self-renewal and the tumorigenicity of PCSCs.

Positive regulators of PCSCs in driving pancreatic cancer formation such as T-box transcription factor 3 **(TBX3)** has been reported to serve as a mediator of PCSCs through an autocrine TBX3-Activin signalling loop, while the expression of TBX3 and ActivinA were significantly repressed by selective compound inhibition of the ALK4 or ALK5 with SB431542 (Perkhofer *et al.*, 2016). In contrast, **miR-17-92 cluster** was a **negative** regulator of PCSCs via targeting Alk4 and TBX3 to confer a CSCs phenotype to non-CSCs (Cioffi *et al.*, 2015). Besides, immunomodulatory cationic antimicrobial peptide 18/LL-37 (**hCAP-18/LL-37**) secreted by tumour-associated macrophages **(TMA)** was strongly expressed in the stroma of advanced primary and secondary PDAC tumors **in response to** PCSCs-secreted Nodal/ActivinA (Sainz *et al.*, 2015).

Thus, these observations together proposed that Nodal/ActivinA as a target for PCSCs therapy (Donahue and Dawson, 2011).

- Anti-tumoral function of Activin-signalling

In 2001, Gloria's study first revealed **somatic mutations in *ACVR1B* could be a suppressor of pancreatic tumorigenesis** (Su *et al.*, 2001). Thus, early pancreatic neoplasia with the high frequency of TGF β -unresponsiveness but low frequency of *SMAD4* mutations may benefit from stimulation of Activin-signalling induced tumor suppression.

Another study using **8 human pancreatic cancer cell lines** (Sui65, Sui66, Sui67, Sui68, Sui70, Sui71, Sui73, and Sui74) shown that **ActivinA inhibits cellular growth by activating the SMAD signal in cell lines with the wild-type *ACVR1B* gene**, but does not activate in cell lines with homozygous deletion of *ACVR1B* gene (Togashi *et al.*, 2014). Further analysis on evaluating the expression levels of $p21^{CIP1/WAF1}$ speculated that p21 may contribute to ActivinA-mediated growth inhibition and cell cycle progression with the presence of both wild-type *ACVR1B* and *SMAD4* genes. Moreover, a TaqMan Copy Number Assay from 29 clinical samples of PDAC shown that 20.7% carried a deletion of *ACVR1B* gene, while 34.5% carried a deletion of the *SMAD4* gene. Thus, these results supported that **the tumor suppressive role of activin signalling through SMAD pathways and p21 is lost following the loss of *AVCR1B*/ALK4**, which leads to an aggressive phenotype of PDAC.

From a more recent study of Gloria's group, the tumor suppressive role of ***ACVR1B*** was further proved (Qiu *et al.*, 2016). They found that generation of AKP mice (*Acvr1b*$^{flox/flox}$*; LSL-Kras*^{G12D}*; Pdx1-Cre*) in the context of oncogenic Kras mutation not only shortened the life span, but also had a tendency in promoting IPMNs precursor development probably mediated by *NOTCH4* signalling pathway. Moreover, the expression of p16 was present in low- to moderate-grade IPMN lesions but absent in advanced grades implying the inactivation of p16 is crucial in driving *ACVR1B*-deficient IPMNs progressing to invasive and metastatic PDAC.

- Activin-signalling mediated cachexia in PDAC

It has been reported that 90% of PDAC patients experience severe weight loss (Parajuli *et al.*, 2018). Togashi *et al.* observed that the bodyweights of mice inoculated with ActivinA overexpressed cells decreased dramatically, and these mice all died at an early stage, suggesting the activin signalling might be involved in cachexia (Togashi *et al.*, 2015). In addition, a recent study from Parajuli *et al.*, also revealed that the transcription factor Twist-related protein 1(Twist1) triggers muscle atrophy through Activin/Myostatin signalling to mediate muscle cachexia. Therefore, targeting of **Activin/Myostatin/Twist** axis may provide a promising therapeutic strategy to prevent cancer-induced cachexia and prolong survival (Parajuli *et al.*, 2018).

2.3.3 Blocking Activin-signalling as a therapeutic option for Cancer

Taken together, these pro-tumoral or anti-tumoral function of Activin in pancreatic cancer occurs via binding and signalling to Activin receptors. Use of TGF β /Activin receptors (ALK4, ALK5 and ALK7) blocking strategies through inhibitors such as PF-03446962 and Dalantercept/ACE-041 (for ALK1), SB505124 (for ALK4, ALK5 and ALK7), LY-2157299 and GW6604 (for ALK5) has been proposed as therapeutic targets for a subset of tumors that exclude pancreatic cancer. While those molecules have shown promising and successful results in Phase I clinical trials, none of them has been yet applied in the clinics, due to the multiple overlapping downstream signalling cascades that they may target and the potential of side- and unwanted effects they may have (Goff *et al.*, 2016; Simonelli *et al.*, 2016; Bendell *et al.*, 2014; Jin *et al.*, 2014; Yoon *et al.*, 2013).

Furthermore, based on previous studies in several mouse models of cancer cachexia, an ActRIIB receptor-based trap (sActRIIB) has been used to treat muscle wasting (Zhou *et al.*, 2010; Busquets *et al.*, 2012). Interestingly, sActRIIB can prevent further skeletal muscle loss but also restore previous muscle mass. Mice treated with sActRIIB experienced an improvement in survival, consistent with the general notion that muscle cachexia represents a key determinant of cancer-related death (Zhou *et al.*, 2010; Tisdale, 2010; Nissinen *et al.*, 2018). However, since the role of activin signalling still remains contradictory, the 'real' benefits of sActRIIB application in pancreatic cancer need to be carefully considered and further evaluated.

2.4 TGFβ- and Activin-signalling in ADM

While almost all the studies mentioned above shed the light on the late stage of PDAC, for me, since I have talked about the importance of ADM as a mechanism of PDAC initiation, it is of great interests to further investigate TGFβ/Activin signalling in ADM. However, both TGFβ- and Activin- signalling are far less studied in this very early stage of pancreatic cancer.

One study suggested the pro-tumoral role of activin signalling in the early stage of PDAC (De Waele *et al.*, 2014). They demonstrated that the transition of the human acinar

cells toward a ductal and mesenchymal phenotype when cultured as monolayers was decreased by inhibition of the TGFβ and activin signalling pathways. Another example is from Bartholin's group, they demonstrated that TGFβ is an ADM inducer and facilitates the development of PDAC in the context of Kras mutation (Chuvin *et al.*, 2017).

On the contrary, Gloria's group reported the tumor suppressive role of *ACVR1B* in early stages of PDAC (Qiu *et al.*, 2016). In this study, they observed formation of ADM, focal chronic inflammatory changes accompanied by proliferation in ductal and stromal cells in pancreas-specific *ACVR1B* deficient mice (*Acvr1b*$^{flox/flox}$*; Pdx1-Cre*).

To conclude, although there are already numerous literatures showing TGFβ signalling is implicated in pancreatic cancer, the studies of Activin-signalling in PDAC are limited and controversial. Therefore, my projet aims to understand the role of ActivinA-signalling in the initiation and progression of PDAC.

3 Senescence

Cellular senescence was first observed by Hayflick and Moorhead in human fibroblasts (Hayflick *et al.*, 1961). In recent years, the importance of senescence in cancer is increasingly recognized. In this chapter, I will start to introduce the definition and main mechanisms inducing cellular senescence. Then I will briefly present the physiopathological impact of senescence. The following two sections will address how senescence contributes to the development of cancer especially in PDAC. Finally, the role of TGFβ superfamily ligands in senescence will be described.

3.1 Cellular senescence

3.1.1 Definition and characteristic of senescent cells

- Definition

Cellular senescence is a stress response that stably blocks proliferation. In general, the two forms of cellular senescence include replicative senescence and premature senescence. Recently, the definition has been renewed considering cellular senescence as a trigger in the remodeling of tissues that acts during normal embryonic development and upon tissue damage. Senescence can be induced by diverse stimuli, it is accompanied with the production of senescence-associated secretory phenotype (SASP) and plays key roles in many physiopathological events including embryonic development, aging and cancer.

Generally, when normal cultured cells enter senescence *in vitro*, they show morphological changes like flatten and enlarged cytoplasm. Whereas *in vivo*, senescent cells keep the normal morphology restricted by tissue architecture. Besides this morphological change, there are a variety of characteristics to distinguish senescent cells from other non-dividing cells [Table 6]. Of note, the stable proliferation arrest and the SASP are two main features of senescent cells. Although none of these markers is specific for all subtypes of senescence, the combination of several of these markers is accepted to define senescence both *in vitro* and *in vivo*.

	Features of senescent cells
β -galactosidase activity	Increased SA- β −Gal activity
Common mediators of senescence	Elevated expression of p16, ARF, p53, p21, p15, p27 and hypophosphorylated Retinoblastoma (RB)
SASP	Prominent secretion of SASP (including soluble signalling factors, secreted proteases, and secreted insoluble proteins/ECM)
DDR markers	Upregulation of ATM, 53BP1, γ -H2AX, CHK2, etc.
SAHF	Nulear foci of constitutive heterochromatin H3K9me3, HP1 γ , etc.
Other markers of senescence	Accumulation of DEC1, DcR2; Induction of SPRY2, Smurf2, WNT16B; Repression of LMNB1
Proliferative markers	The absence of Ki67 or BrdU

Table 6. Common features of senescent cells.

- Markers of senescent cells

The most widely used and standard assay for senescence is called "senescence-associated β -galactosidase" (SA- β -Gal), which was first detected by the blue staining in cells and in skin tissue (Dimri *et al.*, 1995). Its activity at PH 6 is based on the increased lysosomal enzyme in the senescent cells or tissues, which can be easily detected using the chromogenic substrate 5-bromo-4-chloro-3-indoyl β -D-galactopyranoside (X-gal). Besides, it has been demonstrated that SA- β -Gal activity is expressed from the elevated expression of Galactosidase beta 1 (also known as GLB1), which encodes the classical lysosomal β -galactosidase enzyme (Lee *et al.*, 2006). However, limitations of this assay do exist, such as its enrichment in some specific cells types (mature tissue macrophages and osteoclasts) and false positive unrelated to senescence (Kopp *et al.*, 2007). Therefore, SA- β -Gal activity is recommended to identify senescence by the combination of additional markers.

As a stable cell cycle arrest accompanies cellular senescence, the absence of proliferative markers (like Ki67 and BrdU), as well as the elevated expression of tumor

suppressors and cell cycle inhibitors (like p53, p21, p16, etc.), are essential markers for the identification of senescent cells.

DNA damage response (DDR) markers are also the important features to distinguish senescent cells, including upregulation of ATM, 53BP1, γ-H2AX, CHK2, etc. The activation of the DDR pathways is involved in both the initiation and maintenance of senescence in many cases, such as replicative senescence and oncogene-induced senescence (OIS). DNA damage agents, telomere loss and some oncogene expression (such as RAS, RAF, MYC) activate the DDR, which directly activated p53 and its downstream transcriptional target p21 (Harley *et al.*, 1990; d'Adda di Fagagna, 2008; Serrano *et al.*, 1997).

Recent studies have revealed some additional functions of senescent cells. The most noteworthy feature is the senescence-associated secretory phenotype (SASP, also known as the senescence messaging secretome (SMS)) (Coppé *et al.*, 2008). It was first found in human fibroblasts undergoing replicative senescence and then observed in premature senescence (Shelton *et al.*, 1999; Coppé *et al.*, 2008). In general, the SASP components are mainly comprised of three types of proteins: soluble signalling factors (interleukins, chemokines, growth factors and regulators), secreted proteases, secreted insoluble proteins/extracellular matrix proteins (ECM) (Coppé *et al.*, 2010) [Figure 11]. Other SASP components like plasminogen activator inhinibor 1 (PAI-1) and matrix metalloproteinase 3 (MMP3) have been used as senescence markers for a long time (Goldstein *et al.*, 1994; Millis *et al.*, 1992). The regulatory and functional network of SASP will be further discussed in the next section of this chapter.

Another feature of some senescent cells is senescence-associated heterochromatin foci (SAHF), which contain hallmarks of heterochromatin such as trimethylation at lysine 9 of histone 3 (H3K9me3) and heterochromatin protein 1 homologue-γ (HP1γ). Although the foci is not a general phenomenon in all the senescence contexts, SAHF is often formed during OIS (Narita *et al.*, 2003; Di Micco *et al.*, 2011). In addition, other markers that are frequently used to identify senescent cells include the tumor suppressor proteins Deleted in esophageal cancer (DEC1) and Decoy receptor 2 (DcR2), Sprouty2, Smurf2, wingless-type MMTV integration site family member 16B (WNT16B), lamin B1 (LMNB1) and etc..

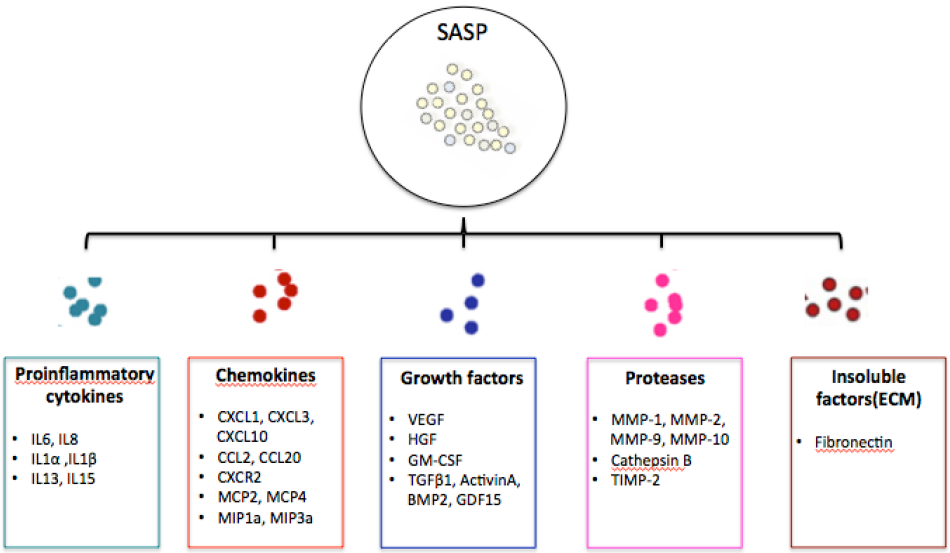


Figure 11. The main SASP components. IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor; HGF: hepatocyte growth factor; MCP: monocyte chemoattractant proteins; MIP: macrophage inflammatory proteins; MMP: matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinases; VEGF: vascular endothelial growth factor (Modified from Muñoz-Espín *et al.*, 2014; Acosta *et al.*, 2013; Kuilman *et al.*, 2008; Coppé *et al.*, 2008).

3.1.2 Mechanisms inducing cellular senescence

Cellular senescence can be induced by diverse stimuli that could be physiological or pathological.

Developmental cues induce senescence via p21 by activating the PI3K and TGF β pathways, while the latter pathway can also be induced by SASP. Another two triggers are polyploidization and cell fusion, which upregulate p21 through Ras-induced activation of the transcription factor early growth response protein 1 (EGR1), and by the DDR and p53/p21 (Muñoz-Espín and Serrano, 2014).

Reported triggering signal of damage-induced senecence include DNA damage and telomere loss, CDKN2A derepression, reactive oxygen species (ROS), oncogenic signalling and tumor suppressor inactivation, and SASP (Muñoz-Espín and Serrano, 2014).

DNA damage, telomere loss and CDKN2A derepression are responsible for initiating the replicative senescence. On the other hand, levels of ROS increase after different types of stresses such as telomere shortening or dysfunction, DNA damage and oncogene

activation, meaning that ROS is implicated in both replicative and premature senescence. Moreover, the engaged pathways causing cells to enter senescence vary depending on the cell type and conditions, although the most commonly activated ones are the p53 and p16 tumor suppressor pathways.

Here, I will focus on the two important mechanisms that are mainly involved in my projet.

- Oncogene induced senescence (OIS)

Oncogene induced senescence (OIS) was first observed by expression of an oncogenic form of human Ras (H-RAS^{V12}) in primary human fibroblasts in 1997. This phenotype showed striking resemblance with replicative senescence that was telomere-independent (Serrano *et al.*, 1997; Wei *et al.*, 1999). From then on, large leaps in understanding this phenomenon have been achieved by a variety of oncogenes *in vitro* and *in vivo*. This means that oncogenic RAS is not the only oncogene cause senescence. Gorgoulis *et al.* summarized a list of about fifty oncogenes that are able to trigger senescence, for example, some mutanted RAS effectors (such as *RAF, MEK, BRAF*), *TGF β, c-Myc, cyclin E, STAT5*, etc (Gorgoulis and Halazonetis, 2010) [Table 7]. In addition to these oncogenes, loss of tumor suppressor genes such as phosphatase and Tensin homolog (*PTEN*), von Hippel-Lindau disease tumor suppressor (*VHL*) and neuroflibromin (*NF1*) can also induce senescence by activating downstream oncogenes (Alimonti *et al.*, 2010; Young *et al.*, 2008; Courtois-Cox *et al.*, 2006). Of note, beside these studies that confirmed the existance of OIS *in vitro*, there is also accumulating evidence since recent years indicating that OIS *in vivo* correspond to a protective mechanism against cancer (Collado and Serrano, 2010; Prieur and Peeper, 2008).

Action	Gene	Pathway — Function
In vivo		
Inactivation	*PTEN* (TSG)	Downregulates PI3K/Akt/mTOR signaling
	VHL (TSG)	Targets HIF for degradation
	Hsp72	Promotes PI3K/Akt (p53-dependent) and ERK (p53-independent) signaling
	c-Myc	Ras signaling effector — transcription and chromatin remodeling factor
	Rb (TSG)	Regulates E2F activity
Activation	*TGFβ*	Promotes Smad signaling
	H-ras^{V12}	Ras signaling
	K-ras^{G12V}	Ras signaling
	N-ras^{G12D}	Ras signaling
	BRAF^{E600}	Promotes Ras signaling
	c-Myc[a,c]	Effector of Ras signaling — transcription and chromatin remodeling factor
	β-Catenin	Promotes Wnt signaling
	Akt	PI3K/Akt signaling
	Rheb	Promotes PI3K/Akt/mTOR signaling
	E2F3	Promotes G1 to S phase — transcription factor
Restoration	*p53* (TSG)	Effector of various signaling pathways — transcription factor
In vitro		
Inactivation	*Rac1*	Modulates Rho signaling
	NF1 (TSG)	Downregulates Ras signaling
	PTEN (TSG)	Downregulates PI3K/Akt/mTOR signaling
	VHL (TSG)	Targets HIF for degradation
Activation	*TGFβ*	Promotes Smad signaling
	INFβ	Promotes STAT signaling — activates p53
	CXCR2 (IL8RB)	Angiogenic CXC chemokine receptor
	Rac1	Modulates Rho signaling
	Smurf2	Upregulation by telomere attrition; promotes p53/pRb senescence
	Runx1, Runx2, Runx3	Transcription and chromatin remodeling factors
	PTEN (TSG)	Downregulates PI3K/Akt/mTOR signaling
	Sprouty 2	Downregulates Ras signaling
	EGFR	Promotes Ras signaling
	H-ras^{V12}	Ras signaling
	N-ras	Ras signaling
	Raf	Ras signaling
	BRAF^{E600}	Promotes Ras signaling
	Mos	Promotes Ras signaling
	MEK	Promotes Ras signaling
	c-Myc[a,b]	Ras signaling effector — transcription and chromatin remodeling factor
	IGFBP3, IGFBP5	Modulates IGF1 signaling pathway
	IGFBP7	Modulates IGF signaling; downregulates Ras signaling
	p38α-D176A, p38γ-D179A	Promotes p38MAPK signaling
	STAT5	Promotes JAK–STAT signaling
	Cyclin E	Activated cyclin-dependent kinase-2: promotes G1 to S phase
	E2F1	Transcription factor: promotes G1 to S phase:
	E2F3	Transcription factor: promotes G1 to S phase:
	Cdc6	Replication licensing factor: promotes S phase progression
	Cdt1	Replication licensing factor: promotes S phase progression
	p16^{INK4A} (TSG)	Cyclin-dependent kinase inhibitor: inhibits G1 progression
	PML	Ras signaling effector; induces p53
	p53^{VP16} (TSG)	Effector of various signaling pathways–transcription factor
	PAI-1	p53 effector
	DEC1	p53 effector

Table 7. Oncogenes and tumor-suppressor genes triggering senescence (Gorgoulis and Halazonetis, 2010).

OIS is considered to be a much more acute event compared to replicative senescence, usually referred as “stress-induced senescence” (Campisi and d'Adda di Fagagna, 2007). It has been suggested that OIS acts as a good initial barrier to cancer when cell damage is under repaired. For example, as a common oncogene, activated Ras was reported to trigger

an initiation of proliferation, but followed by an accumulation of p53/p21 and p16 proteins with the participation of the DDR and ARF. Further studies showed that activated Raf-MEK-ERK by oncogenic Ras sequentially activated MKK3/6(also known as MAPKK3 and MAPKK6)-p38 leading to senescence with the elevated p53 and p16, on the other hand, this activated MEK-ERK pathway can also promote cell proliferation through transcriptional activator protein 1 (AP-1) (Serrano *et al.*, 1997; Wang *et al.*, 2002). Therefore, inactivation of either p53 or $p16^{INK4A}$ can bypass Ras-induced senescence in normal human and mouse cells in culutre (Serrano *et al.*, 1997). Furthermore, OIS can also induce a DDR associated with DNA hyper-replication and/or ROS [Figure 12].

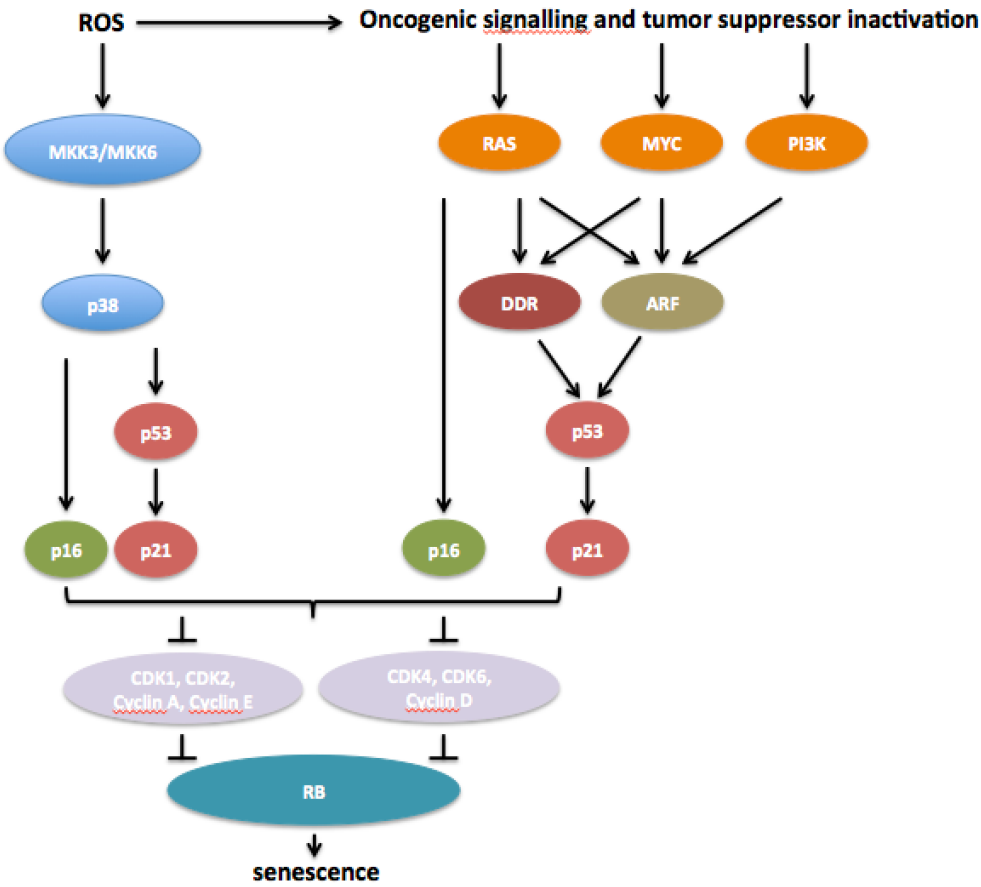


Figure 12. Molecular pathways of OIS.

- Senescence-associated secretory phenotype (SASP)

As previously described, senescent cells secrete a variety of senescence-associated secretory phenotype (SASP) which can be classified into five groups [Figure 11]. The SASP reinforces the senescent phenotype both in an autonomous way through an autocrine feedback loop and on the surrounding cells by a paracrine manner or non-autonomous way, resulting in creating an inflammatory microenvironment that associated with the elimination of senescent cells (Acosta *et al.*, 2008; Rodier, 2013). Besides, it is also a key feature to distinguish senescent cells from non-senescent cells such as quiescent cells and terminally differentiated cells.

Different cell types and triggers of senescence secrete different SASP factors. The most common SASP factors are known as the pro-inflammatory cytokines such as interleukin-1α (IL1α), IL-1β, IL-6, and IL-8 (Perrott *et al.*, 2017; Kuilman *et al.*, 2008). IL1α has also been proposed as a key upstream effector of SASP. Kuilman *et al.* revealed that senescent cells can directly affect adjacent cells through IL-6. Besides, several studies showed that SASP especially TGFβ can trigger senescence in neighbouring cells in a paracrine manner (Acosta *et al.*, 2013; Nelson *et al.*, 2012; Hubackova *et al.*, 2012). Furthermore, the SASP factors are also reported to promote a proregenerative response via the induction of cell plasticity and stemness or to promote proliferation and epithelial-to – mesenchymal transition (EMT) under some certain contitions (Ritschka *et al.*, 2017; Kuilman *et al.*, 2008).

It is accepted that p16 and p53/p21 tumor suppressor pathways are the two most commonly activated ones during cellular senescence. However, recent findings suggest that SASP induction could be blunt without affecting these two pathways. Diverse stresses such as OIS and DNA damage can induce SASP through multiple mechanisms including transcriptional activation, stabilization of transcripts, and chromatin remodeling (Watanabe *et al.*, 2017) [Figure 13]. SASP induction is mainly mediated by nuclear factor-κB (NF-κB) and CCAAT/enhancer binding protein-β (CEBPβ) signalling (Chien *et al.*, 2011; Herranz *et al.*, 2015). p38MAPK and mTOR signalling have been proposed to induce SASP factors through pro-inflammatory cytokines like IL1α and activated NF-κB signalling pathway (Hubackova *et al.*, 2012; Freund *et al.*, 2011). On the other hand, DDR also plays a crucial role in SASP induction. For example, the transcription factor GATA binding protein 4 (GATA4) was reported to function as un upstream activator of NF-κB to induce SASP factors via the DDR kinases ataxia telangiectasia mutated (ATM) and

ataxia telangiectasia and Rad3-related (ATR), rather than p53 or p16^{INK4a} (Kang *et al.*, 2015). In addition, the expression of SASP genes can also be modulated by chromatin reorganization, which includes several epigenetic regulators such as G9a and GLP histone methyltransferases, high mobility group box 2 (HMGB2), mixed-lineage leukemia 1 (MLL1) and bromodomain-containing protein 4 (BRD4) (Takahashi *et al.*, 2012; Aird *et al.*, 2016; Capell *et al.*, 2016; Tasdemir *et al.*, 2016). These secreted SASP factors further trigger senescence by modulating the upregulation of cell cycle inhibitors (p21, p15, p27) through TGF β -SMAD pathway independent of p53 or p16 (Muñoz-Espín and Serrano, 2014) [Figure 14].

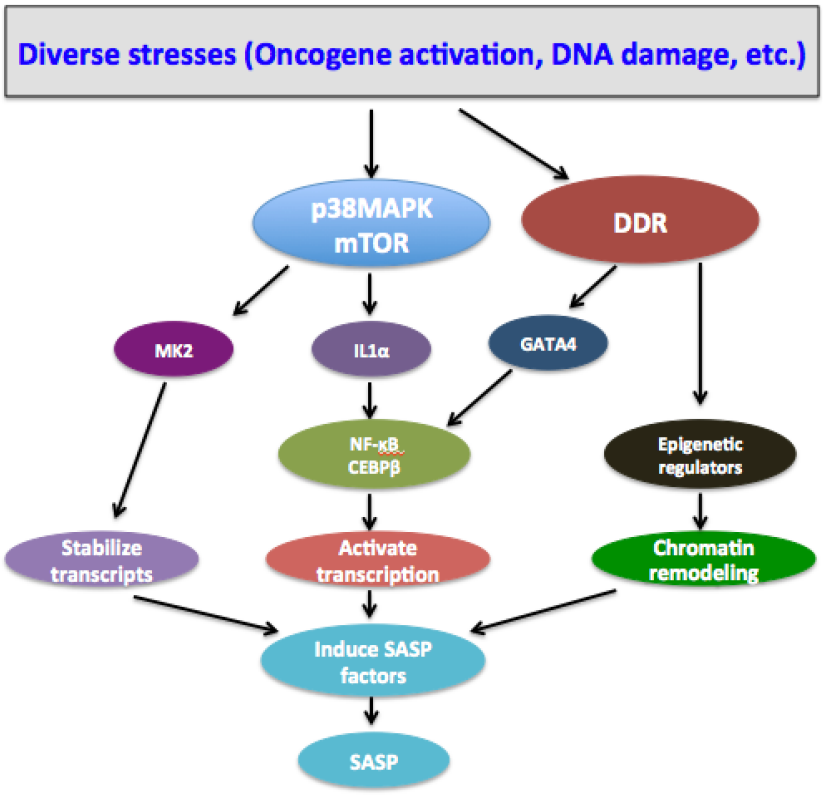


Figure 13. Regulatory pathways of SASP (Modified from Watanabe *et al.*, 2017).

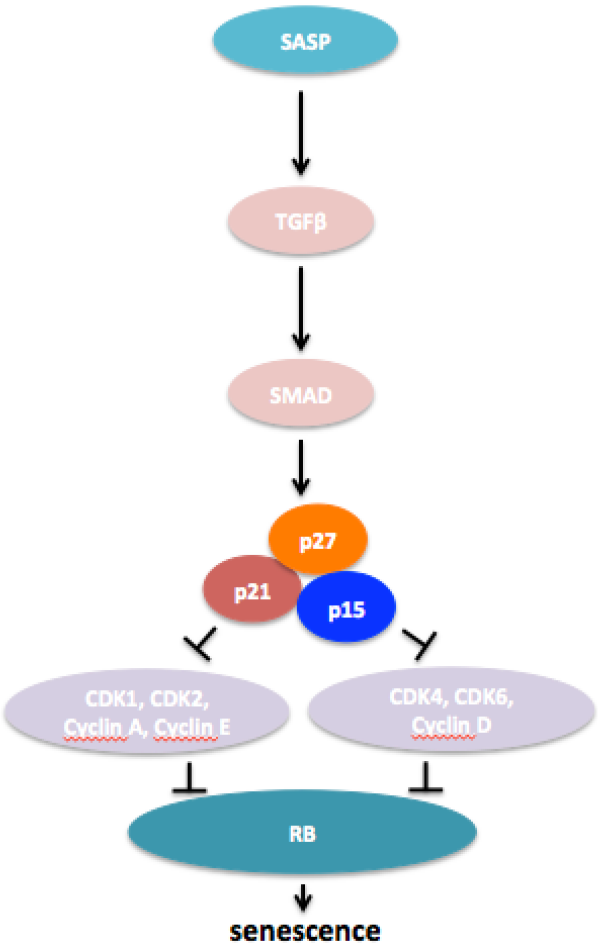


Figure 14. Molecular pathway of SASP mediated senescence [modified from Muñoz-Espín and Serrano, 2014].

3.2 Pathophysiological impact of cellular senescence

Cellular senescence is driven by a wide range of stimulus leading to diverse outputs. Biological consequences in short term of senescence include embryonic development, wound healing and tissue regeneration, tumor suppression, while in long term of senescence comprise tissue degeneration, aging and aging-associated diseases and tumor promotion related to the persistence of senescent cells and chronic inflammation (Burton *et al.*, 2014; Watanabe *et al.*, 2017) [Figure 15].

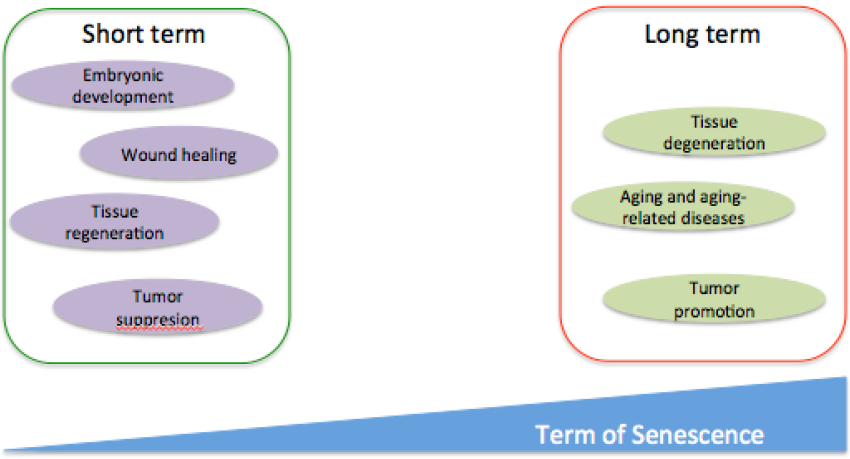


Figure 15. Short term and long term of senescence. Senescence can display benefical or detrimental effects depending on tissue or cell types and duration of senecent cells.

3.2.1 Embryonic development

Developmental senescence is a conserved feature during embryogenesis as it can be observed across vertebrates such as mouse and human. In embryonic development, cellular senescence plays crucial roles in shaping organogenesis, in the growth and integrity maintaining of tissue, and in regulating growth of placenta.

It has been reported from both Munoz-Espin's and Storer's studies showing the senescence-positive structures in the apical ectodermal ridge (AER) of the limbs, the mesonephric tubules during mesonephros involution, the endolymphatic sac of the inner ear, the regressing interdigital webs and the closing neural tube (Storer *et al.*, 2013; Muñoz-Espín *et al.*, 2013). Interestingly, while the senescent cells in these structures are characterized by several common features of senescence including SA- β -Gal activity, the absence of Ki67 and BrdU, increased heterochromatin markers H3K9me3 and HP1 γ , and elevated expression of cell cycle inhibitors (p21, p15, p27), they express neither DDR markers nor typical SASP cytokines. And the senescence in this context is induced through TGF β /SMAD/p21 or PI3K/FOXO/p21 pathways independent of p16 and p53.

Placental syncytiotrophoblasts also undergo senescence which is triggered by cell-cell fusion. The endogenous retroviral protein ERVWE1 is a cell fusion protein functioning in the formation of the placental syncytiotrophoblast, which shows multiple senescence

features such as SA-β-Gal staining, DDR markers, p53, p21, and p16 (Chuprin *et al.*, 2013). Besides, these cells also express SASP factors that further enhance senescence phenotype by modulating the surrounding microenvironment (Gal *et al.*, 2014).

Although senescence is common during embryogenesis with multiple features, its physiological function needs to be further discovered.

3.2.2 Wound healing and tissue repair

Cellular senescence has been implicated to participate in pathological wound healing with the induction of myofibroblasts and the secretion of SASP (Ritschka *et al.*, 2017; Demaria *et al.*, 2014; Tomasek *et al.*, 2002). Thus, it limits multiple tissue damage and initiates the repair as well as returns the tissue to its normal state. Among the tissue damage, most of the literatures focus on the liver and skin, which are the two big and important regenerative organs in the body.

Demaria *et al.* revealed that p16 positive senescent fibroblasts and endothelial cells appear early at the injury site due to a production of SASP factor called platelet-derived growth factor AA (PDGF-AA) and the elimination of these cells will delay the healing in mice (Demaria *et al.*, 2014). Study from Krizhanovsky *et al.* demonstrated that carbon tetrachloride ($CC1_4$)-induced chronic liver damage and fibrosis in mice can be restricted through the accumulation of activated senescent stellate cells and the upregulation of SASP modulating the tissue microenvironment (Krizhanovsky *et al.*, 2008). This suggested that senescence helps to repair the liver fibrotic scar. Another study from Keyes's group, in which they collected the conditioned medium (CM) from vector-infected growing cells (VCM) or OIS keratinocytes (OIS-CM) and put it to proliferating newborn primary mouse keratinocytes (PMK) during a two-day period, they then observed a prominent increase of stem cell genes and an induction of skin regeneration associated with transient exposure of SASP from the OIS-CM (Ritschka *et al.*, 2017). On the other hand, they also uncovered that prolonged exposure to SASP will lead to the response of paracrine senescence, the arrest of cell-intrinsic senescence and the attenuation of regenerative capacity. This gives a good example for us showing both the beneficial and deleterious function of senescence during tissue repair. In addition to the expression of p16 and SASP factors, NF-κB

activation is also reported to be crucial for healing (Ambrozova *et al.*, 2017; Yang *et al.*, 2017).

Although senescence plays important roles in wound healing and tissue repair, it needs further investigation in many aspects, for example, its role in different wound healing-related diseases.

3.2.3 Aging and aging-associated diseases

Aging is characterized by the progressive loss of tissue and organ function over time (Flatt, 2012). Many studies have shown that senescence is closely linked to aging. Several reasons are responsible for the accumulation of senescent cells in aged tissues, including the persistent existence of senescence-inducing stresses, defects in clearance of senescent cells by immune system and paracrine induction of senescence through SASP (van Deursen, 2014). This phenomenon can also be observed by the use of *in situ* detection methods including multiple senescent cell features such as high levels of p16, p21, IL-6 and SA-β-Gal activity.

Now that aging initiates heterogeneously across multiple organ systems and finally results in tissue dysfunction, therefore, it addresses us another question that how these accumulating senescent cells induce age-related tissue dysfunction. Two possible mechanisms are considered to be involved: one is the SASP based mechanisms, and the other is the decline of regenerative stem and progenitor cells due to senescence itself (van Deursen, 2014). Here, I will give some examples of the impact of senescence on aging-associated diseases.

In cardiovascular disease, ablation of senescent vascular smooth muscle cells (VSMCs) and macrophages seems to improve the plaque stability and decrease the plaque formation (Uryga and Bennett, 2016; Childs *et al.*, 2016). Several senescent markers such as SA-β-Gal activity, p16, p38, IL-1β increased in diabetic mice, suggesting a possible link between senescence and diabetes (Sone and Kagawa, 2005; Zeggini *et al.*, 2007; Dinarello *et al.*, 2010). A decreased Glomerular filtration rate (GFR) usually results in glomerulosclerosis and nephron atrophy in aging people, which are related with upregulation of p16 and p53 (Melk *et al.*, 2004). Targeting these senescent cells might improve renal function and protect against glomerulosclerosis (Sturmlechner *et al.*, 2017).

The role of cellular senescence is controversial during the development of idiopathic pulmonary fibrosis (IPF). SASP components secreted by senescent cells in IPF act either antifibrotic or profibrotic (Craig *et al.*, 2015; Schafer *et al.*, 2017). It has been reported that liver fibrosis and nonalcoholic fatty liver disease are associated with senescence by the presence of senescent hepatocytes (Pellicoro *et al.*, 2014; Hardy *et al.*, 2016). The benefits from accumulation of senescent cells in liver also remain contradictory, as reported to restrict fibrosis in one study and increase hepatic steatosis in another (Krizhanovsky *et al.*, 2008; Ogrodnik *et al.*, 2017). In age-related cachexia, the elimination of senescent cells can increase adiposity and improve metabolic function in aging mice (Baker *et al.*, 2016; Xu *et al.*, 2015; Berry *et al.*, 2017).

3.3 Role of senescence in cancer

Senescence was linked to tumorigenesis both in mice and in humans more than ten years ago (Braig *et al.*, 2005; Collado *et al.*, 2005). Although accumulating evidences support senescence plays **a tumor suppressive role** during tumorigenesis via both cell-intrinsic and cell-extrinsic mechanisms, it is worth mentioning that senescence can also play **a pro-tumoral role** under some conditions.

3.3.1 OIS as a safe-guard program

Several studies have confirmed the role of senescence in tumor suppression. **Firstly, the original analysis of senescence in tumors is the observation of senescent tumor cells in a number of premalignant tumor stages.** In murine studies**,** abundant senescent cells were identified in benign lesions of the prostate, in lung adenomas, in lymphocytes, in hyperplasias of the pituitary gland, in melanocytic lesions, and in mammary gland (Chen *et al.*, 2005; Collado *et al.*, 2005; Braig *et al.*, 2005; Lazzerini *et al.*, 2005; Ha *et al.*, 2007; Sarkisian *et al.*, 2007). Similarly, in human tumors, senescent cells can also be obtained in benign lesions of human naevi and prostate (Michaloglou *et al.*, 2005; Chen *et al.*, 2005). These together suggest that senescence restrains tumor progression by the early presence of senescent cells in the premalignant stages during tumorigenesis and could be a promising identified marker for tumor staging.

Secondly, the existence of OIS proceeds a protective mechanism against cancer. The conduction of this protection involves several key points including levels of oncogene activity, tumor suppressor pathways, and tumor suppressive upsteam and downstream of oncogenes. Numerous studies have focused on Ras oncogene showing that different levels of Ras activity determine different outcomes. Normal or low expression of oncogenic Ras can trigger hyperplasias but do not produce tumors, while overexpression of oncogenic signalling leads to the formation of premalignant tumors as well as the induction of senescence (Tuveson *et al.*, 2004; Sarkisian *et al.*, 2007). Besides, the oncogenic Ras signalling becomes tumorigenic when the negative regulators are deleted or when the expression of the oncogenes is upregulated and exceeds that of the negative regulators. Of note, various cellular context and various oncogenes (such as PI3K, E2F, HIF1α) might have different effects (Guerra *et al.*, 2003; Michaloglou *et al.*, 2005).

After aberrant expression of oncogenic Ras in normal primary cells, the antiproliferative power of senescence is initiated through the two main tumor suprressive pathways---p16 and p53. It was reported that p53-mediated senescence-like arrest *in vitro* is reversible while the concomitant activation of p16 and p53 mediates an irreversible senescence-like arrest (Beauséjour *et al.*, 2003). However, these two pathways are not the only pathways linked with tumor suppression. For example, detectable senescence was still induced in the absence of p53 or p16 (Xu *et al.*, 2008; Dhomen *et al.*, 2009). Besides, it is revealed that other cell cycle regulators such as p27 coded by CDKN1B can also be involved in OIS associated tumor suppressive mechanism (Majumder *et al.*, 2008).

In addition to these downstream oncogenes that give response to the activation of oncogenes and induce senescence, it needs to be mentioned that another category of tumor suppressors called upstream of oncogenes prevents excessive OIS whereas their ablation in normal cells triggers senescence such as PTEN, VHL, NF1, RB as we have described before. The next step in OIS antitumorigenic process will be described in the following part.

3.3.2 Ambigous role of SASP in cancer

- Antitumorigenic effect

Diverse stress-induced SASP especially by OIS contributes to tumor suppression either in a cell-autonomous or in a non-cell-autonomous manner. Upregulation of multiple SASP components can drive cell cycle arrest, as well as attract and activate immune cells to facilitate the immune surveillance (Xue *et al.*, 2007; Kuilman *et al.*, 2008; Kang *et al.*, 2011). Besides, the SASP was also reported to have an implication on tumor suppression by paracrine senescence. For example, a recent study from Acosta *et al.* demonstrated that several TGF β family ligands are upregulated during senescence and it can mediate paracrine senescence by regulating $p21^{CIP1}$ and $p15^{INK4b}$ (Acosta *et al.*, 2013).

- Protumorigenic effect

On the other hand, in addition to the tumor suppressive role, senescence might also promote cancer progression with the long-term presence of senescent cells affecting both the tumor cells and the tumor microenvironment. Senescent cells are responsible for tumor progression by reinforcing the proliferative potential of cancer cells or contributing to EMT (Krtolica *et al.*, 2001; Coppé *et al.*, 2008). Besides, aged tissues with the accumulation of senescent cells can create a supportive niche for cancer progression through a certain subset of SASP secretion (Coppé *et al.*, 2010). According to these views, elimination of senescent cells will delay the onset in tumor formation or reduce the incidence of metastasis (Baker *et al.*, 2016; Demaria *et al.*, 2017).

3.3.3 Senescence-associated stemness in cancer

In recent years, with the cognition of some SASP components such as IL6 and IL8 that are accociated with CSCs, people start to notice the potential link between senescence and stemness in cancer (Iliopoulos *et al*., 2011; Kim *et al*., 2013; Charafe-Jauffret *et al*., 2009). One example is from Ortiz-Montero *et al*., they demonstrated that pro-inflammatory SASP factors IL6 and IL8 induce a self- and cross-reinforced senescence/inflammatory milieu responsible for the emergence of epithelial plasticity and stemness features conferring more aggressive phenotypes to a breast cancer cell line (Ortiz-Montero *et al*., 2017). Later on, Milanovic first gave a clear identification to explain this relationship called senescence-asscociated stemness (SAS) (Milanovic *et al*., 2018). Most studies by far support that senescence promotes cancer stemness through the pro-inflammatory or

pro-fibrotic SASP factors. Liou *et al*. demonstrated that IL13 produced by a subset Dclk1+ (known as a marker for tuft cells or stem cells) cells in pancreatic low-grade lesions promotes fibrosis through a macrophage-mediated inflammatory responses (Liou *et al*., 2017). A more recent study also revealed that phospholipase D2 secreted by colon tumor cells can increase cancer stemness through the communication between a series of pro-inflammatory SASP factors and microenvironment (Muñoz-Galván *et al*., 2019). However, since senescence plays either an anti-tumoral role or a pro-tumoral role depending on the context including different cell types, variable stimulus of senescence, as well as the duration and composition of SASP, it is worth further investigating the role of senescence on cancer stemness.

3.4 Cellular senescence in PDAC

3.4.1 OIS in PDAC

It is well-known that $Kras^{G12D}$ is the most common oncogene during the development of PDAC, while at the meantime, it also triggers the OIS in the early premalignant lesions diminishing its tumorigenic properties. More than 10 years ago, senescent cells were first observed in pancreata of Kras mutated mice with low-grade PanINs staining positive for p16, Dec1, and DcR2 (Collado *et al.*, 2005). Caldwell *et al.* later demonstrated not all mutant Kras-expressing cells become senescent as about one-tenth of cells within PanIN-1 lesions showing an increased SA-β-Gal activity (Caldwell *et al.*, 2012). Similarly to the common feartures of senescent cells in other tissues, these SA-β-Gal positive cells also show a non-proliferative property and display an activation of p16 and p53/p21 pathway mediated by OIS (Caldwell *et al.*, 2012; Eser *et al.*, 2013). Furthermore, it is worth mentioning that a subset of proliferative cells without SA-β-Gal staining coexist with these senescent cells in the same PanINs lesions, suggesting they might coordinate for the fate of the lesions. However, OIS becomes less or rarely detectable in PanIN2/3 or in PDAC (Ikezawa *et al.*, 2017). By contrast, limited studies revealed senescent cells in human PDAC probably due to the fact that most human PDAC are in very advanced stage, although p16-positive cells were detected within some PanINs lesions nearby the PDAC (Guerra *et al.*, 2011).

Similar to the role of senescence in other cancers, senescence has also been reported to function as a barrier in pancreatic cancer. The two key senescence effectors p16 and p53 are also the two most commonly mutated genes in PDAC with Kras mutation. Moreover, inactivation of p16 or p53 leads to the acceleration of PDAC progression and the promotion of metastasis (Aguirre *et al.*, 2003; Hingorani *et al.*, 2005). However, the genetic inactivation of p16 and p53 usually appears in the late stage of PDAC. **Thus, as I previously described, the upstream or downstream of oncogenes and diverse stress-induced SASP can also contribute to the anti-tumoral role of senescence in PDAC.** From Kennedy's study, they demonstrated that the activation of PTEN/PI3K/AKT pathway inhibits Kras OIS and accelerates pancreatic tumorigenesis, therefore inhibition of this pathway can be exploited as a pro-senescence anti-cancer therapy (Kennedy *et al.*, 2011). Another example from the modulation of downstream of oncogenic Kras is the liver kinase B1 (LKB1), which encodes a serine/threonine kinase that activates a number of downstream kinases, cooperates with Kras to suppress PDAC associated with p21-dependent growth arrest (Morton *et al.*, 2010).

Although all these observations above suggest OIS excerts a protection against pancreatic tumorigenesis, there are some evidences showing its dark side in PDAC. For example, under the condition of pancreatitis, mutated Kras performs an inhibitory effect on senescence in normal pancreatic duct epithelium (PDEC) through Kras-Twist-p16 pathway, that is, the expression of Twist may allow oncogenic Kras-expressing PDEC to escape senescence at an earlier stage when the p16 locus has not been disrupted yet (Lee *et al.*, 2010). In another study, OIS within low-grade PanINs was inhibited by pancreatitis-induced inflammation displaying a decreased expression of p16 and accelerating PDAC progression (Guerra *et al.*, 2011).

3.4.2 Known SASP factors in PDAC

In addition to the upstream and downstream modulation of OIS, the SASP secreted by the OIS cells from tumor or stroma also excerts an anti-tumoral function in PDAC. In Algül's study, they revealed that the activation of the C-X-C chemokine ligand 1 (CXCL1) through its receptor CXCR2 expressed in PanINs lesions promotes OIS and attenuates pancreatic tumorigenesis (Lesina *et al.*, 2016). Furthermore, the SASP also regulates a paracrine effect in senescence, Acosta *et al.* displayed multiple components of the SASP,

such as chemokines, TGF β family ligands or VEGF from a secretome analysis of the OIS cells, are involved in pacacrine senescence (Acosta *et al.*, 2013). They further reinforced their conclusion by showing the blunted tumor suppressive function in PDAC via the inhibition of TGF β signalling pathway.

Similarly to the dark side of OIS in PDAC, a number of SASPs have also been reported to promote PDAC progression. IL1α as a SASP factor and a key upstream regulator of SASP, its overexpression cooperates with mutant Kras through activation of NF-κB leads to a poor survival in PDAC patients (Ling *et al.*, 2012). Therefore, blocking IL1α / NF-κB pathway leads to a blunted inflammatory response and an improved outcome both in murine and in human PDAC (Zhuang *et al.*, 2016; Ling *et al.*, 2012). Another SASP component IL-6, induced by OIS, was revealed to serve as a promoter for tumorigenic growth in some human pancreatic cancer cell lines in a paracrine manner, such as Capan-1 and CFpac-1 (Ancrile *et al.*, 2007). Liou *et al.* reported that IL-13 secreted by a subset of cells at pancreatic low grade lesions promotes fibrosis and mediates tumorigenesis by altering macrophage-mediated inflammatory responses (Liou *et al.*, 2017). Yet, since SASP comprises a wide range of secreted proteins, the role of SASP in the context of PDAC remains extensively unknown and still needs to be further investigated.

Another important thing worth paying attention is the impact of senescence on PCSCs and their niches. While some previous studies showed that induced senescence in mouse mammary epithelial stem cells or in human mesenchymal stem cells decrease the risk of developing cancer (Boulanger and Smith, 2001; Serakinci *et al.*, 2004), some others debated that senescence in stem cells promote tumorigenesis through the exhaustion of stem cells pools or the disruption of stem cell microenvironment (Rudolph *et al.*, 1999; Wong and Collins, 2003). In pancreatic cancer, it is reported that PSCs also called stromal resident fibroblasts create a niche for PCSCs which further promote the self-renewal and invasiveness of PCSCs (Lonardo *et al.*, 2012). Unfortunately, limited studies focus on the relationship between senescence and PCSCs and their niches. One example from Fitzner *et al.* showed that a transient presence of senescent PSCs in the context of non-Kras mutated pancreatitis helps to reduce pancreatic fibrogenesis, whereas the long existence of senescent PSCs reelevates fibrogenesis associated with inflammation (Fitzner *et al.*, 2012).

A more recent study, by taking advantage of Panc-1 cell line with Kras mutation, they demonstrated that senescent PSCs promote pancreatic tumor cell growth, invasion, and macrophage phenotype transformation with an elevated level of IL-8, CXCL1 and CXCL2 (Shao *et al.*, 2019). Therefore, it is still worth further digging into the role of senescence on the determination of PCSCs in the development of PDAC.

3.5 Role of TGF β superfamily ligands in senescence

3.5.1 Profibrotic role of TGFβ signalling

TGFβ signalling as a profibrotic cellular processes has been well recogniazed. For example, in the development of lung fibrosis, ROS can enhance TGFβ signalling and promote fibrosis (Gonzalez-Gonzalez *et al.*, 2017). TGFβ signalling can eliminate the activities of anti-fibrotic protein and potentiates renal fibrogenesis (Yin *et al.*, 2017). Besides, its profibrotic role has also been reported in other systems such as skeletal muscle and liver (Parker *et al.*, 2017; Oh *et al.*, 2012).

Of note, while members of the TGFβ-signalling family have been associated with a profibrotic senescence phenotype in opposition to the pro-inflammatory SASP mediated by NF-κB, ligands of the TGFβ-superfamily have been further proposed to be important actors of the paracrine senescence mediated by Kras (Hoare *et al.*, 2016; Acosta *et al.*, 2013).

3.5.2 Identification of novel TGFβ superfamily SASP

As previously described, numerous studies point TGFβ plays a crucial role in SASP mediated senescence in different conditions. In recent year, it has been mentioned that not only TGFβ, other family ligands such as ActivinA, GDF15, BMP6, and BMP2 engage in paracrine senescence by regulating $p21^{CIP1}$ and $p15^{INK4b}$ (Acosta *et al.*, 2013). As ActivinA is one of the most important ligand in TGFβ superfamily and the role of ActivinA-signalling involving in PDAC, it is worth digging out the role of this ligand in senescence.

Besides, the idea that ActivinA as a candidate SASP component induced by Kras OIS arises from a study showing *INHBA* gene is a target of activated oncogenic Kras in pancreatic duct cells (Qian *et al.*, 2005). However, compare to TGFβ, limited studies pay attention to ActivinA-mediated pathway in Kras OIS and most of them are focused on *in vitro* part. One *in vitro* study demonstrated that autocrine and paracrine ActivinA mediated hepatocyte growth inhibition and cellular senescence through $CDKN2B/p15^{INK4b}$ (Haridoss *et al.*, 2017). In a neuroblastoma xenograft model, ActivinA exhibited an inhibitory effect on endothelial cell proliferation by increasing expression of p21 through ALK4/SMAD cascades (Panopoulou *et al.*, 2005). In the analysis of human pancreatic cancer cell lines, ActivinA inhibits cell growth through an intact ALK4/SMAD/p21 pathway (Togashi *et al.*, 2014). Moreover, loss of p21 has been reported to increase ADM formation and senescence in the context of pancreatitis, whereas the inhibition of p21 accelerates Kras induced PDAC formation and overcomes Kras induced OIS at the same time (Grabliauskaite *et al.*, 2015; Morton *et al.*, 2010).

Therefore, these observations suggest a potential link between Activin-signalling and senescence. And it is also of great interests for my projet to have a deep look into the networks of both of them in the early and late stage of pancreatic cancer.

OBJECTIVES

In recent years, the role of Activin-signalling has been highlighted during pancreatic tumorigenesis with the use of *in vivo* and *in vitro* experimental models. Mutated genes such as *SMAD4, ACVR1B* and *ACVR2A* within this signal are known to have an important role in PDAC. Among them, the important Activin receptor---*ACVR1B*/ALK4 has been emphasized. Most of the literatures are dedicating to the function of Activin-ALK4 signalling in the late stage of this deadly disease. However, the studies are limited and controversial.

As more and more recent literatures support the hypothesis that acinar cells are the cell origin for precursor and cancerous lesions, ADM is believed to be an initial process of great importance during PDAC development. Oncogenic Kras contributes to the formation of ADM and also trigger senescence in ADM and low-grade PanINs, which acts as a barrier in pancreatic cancer, accompanying with an emerging secretion of various SASP.

In this case, it is of great interests for me to understand the exact contribution of Activin-signalling through ALK4 in the early stage and progression of PDAC during my PhD study. Therefore, **the objective of my thesis** was initially to:

i) study the consequence of the loss of Alk4 in the initiation of PDAC based on a Kras mutated mouse model,

ii) identify a candidate ligand for Alk4 function and study its cellular function in the onset of PDAC, and

iii) dissect the autocrine and paracrine functions of ActivinA-signalling through ALK4 in the progression of PDAC.

RESULTS

Part 1 ActivinA Expression during Acinar to Duct Metaplasia promotes a Senescent Program that limits Pancreatic Cancer initiation in Mice (Manuscript)

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Short Title: ActivinA limits Pancreatic Cancer Progression

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Abbreviations: PDA, Pancreatic Ductal Adenocarcinoma; ADM, Acinar-to-Ductal Metaplasia; OIS, Oncogene-Induced Senescence; SASP, Senescence Associated Secreted Phenotype; IHC, ImmunoHistoChemistry; IF, Immuno Fluorescence; PanINs: Pancreatic intraepithelial neoplasia; IPMNs, Intraductal papillary mucinous neoplasms; FFPE, Formalin-Fixed Paraffin Embedded.

Conflicts of interest: The authors disclose no conflicts.

Author Contributions: PB and YZ designed research studies. DG, MP, MC, GC conducted experiments. PB, YZ, DG, MP, MC, and AH acquired data. YZ, AH, SV, RT, JF, DB, OR and PB analysed data and discussed results. SV and RT provided animal material and transcriptomic data. PB wrote the manuscript.

ABSTRACT

Background & Aims: Pancreatic Ductal Adenocarcinoma (PDA) represents one of the most aggressive cancer. Understanding mechanisms that drive pre-neoplastic pancreatic lesions is therefore important to improve early-diagnostic and -therapeutic. Mutations and inactivation of ALK4-receptor functions has been demonstrated to favour PDA onset. Surprisingly, little is known about the ligand driving ALK4-signalling in pancreatic cancer and how this signalling limits the onset of neoplastic lesions. Our work was aiming at identifying the ligands driving ALK4-signalling in PDA and further studying their contribution in Acinar-to-Ductal Metaplasia (ADM). **Methods:** ActivinA expression was identified through data-mining and validated on human-TMA and mouse pancreatic-sections. 3D-cultures of pancreatic acinar-cells and histological analysis of $Kras^{G12D}$ pancreas-expressing mouse-models were used to dissect the role of ActivinA in ADM and PDA initiation. Consequences of ActivinA-signalling inhibition were studied in mice and cell-cultures through the use of soluble Activin-typeIIB-Receptor (sActRIIB-Fc) or conditional ALK4-Knockout. **Results:** Our work pinpoints that ActivinA, which is absent in normal acinar-cells, is strongly induced in ADM-lesions promoted by pancreatitis or $Kras^{G12D}$ in mouse and human. More importantly, our results demonstrate that ActivinA-expression in ADM contributes to the cellular senescence program induced in those precursor lesions. Blocking ActivinA-signalling through the use of sActRIIB-Fc or ALK4-KO mice expressing $Kras^{G12D}$ results in a senescence-escape associated with reduced p21 and γH2AX expression, an increased proliferation and the onset of larger early lesions that adopt later a cystic morphology. **Conclusions:** Here, our work identifies ActivinA as a tumour-protecting SASP-factor, produced by ADM/PanIN senescent-cells, that limit - *through ALK4* - their proliferation and expansion into highly proliferative lesions.

INTRODUCTION

Pancreatic Ductal Adenocarcinomas (PDA) have one of the worst outcomes among all cancers with a median survival around 6 months and a 5-year survival rate < 8% [1]. While a large number of therapeutics for PDA has emerged over the last decade, their efficiencies remain relatively limited and proper approaches to circumvent the late diagnostic of those tumours are still lacking [2]. Studies exploring mechanisms and mutations that lead to the onset of PDA have confirmed the major role of Kras activating mutations in driving the formation of Acinar-to-Ductal Metaplasia (ADM) and Pancreatic Intraepithelial Neoplasia (PanINs) that precede the development of PDA [3, 4]. While the progression of these lesions to PDA requires additional mutations, such as p16, p53 and others, the latency that takes place prior PanINs evolve in aggressive and invasive tumours supports the existence of mechanism limiting the transformation of neoplastic cells. Interestingly, whereas activating mutations of Kras drive the onset of PDA [5], Kras can also promote Oncogenic Induced Senescence (OIS), a safeguard program limiting the proliferation and initiation of Cancer lesions [6, 7]. The implication of OIS in governing pancreas tumorigenesis has already been confirmed [8-12] and the use of animal model expressing $Kras^{G12D}$ supports the existence of a senescence tumour-suppressive program that limits the expansion of Kras-mutated cells [13, 14]. Beside the growth-arrest OIS promotes on cancer-initiated cells, senescence cells are capable of producing and secreting a complex series of cytokines and molecules which are referred as the Senescence-associated secretory phenotype (SASP) [15]. Up to date, several SASP protein have been identified among which an important number are driving pro-inflammatory responses [16]. While the exact role of SASP-cytokine and pro-inflammatory component in PDA initiation is still debated [17, 18], recent evidences support the importance of SASP molecule such as CXCL1 in pancreatic tumorigenesis in tumour surveillance [18].

Among SASP candidates potentially involved in paracrine senescence, a number of TGFβ-superfamily ligands – *TGFβ1, INHBA, BMP2 and GDF15* – have been pointed through

secretome analysis of cell undergoing OIS [19]. Interestingly the *INHBA* gene – *encoding for ActivinA* – is a target of Kras oncogenic activation in pancreatic duct cell [20] suggesting a potent implication of ActivinA as a candidate SASP induced by Kras OIS in pancreatic neoplastic lesions. Interestingly, while ablation of the Activin-cognate receptor ALK4 accelerates the onset of PDA and favours the development of intraductal papillary mucinous neoplasms (IPMNs) in mice harbouring *Kras*^{G12D} mutations [21, 22], little is known about the contribution of Activin-signalling through ALK4 in the earliest stage of Kras-driven pancreatic cell transformation nor the implication of this pathway in Kras-OIS within low grade pancreatic neoplastic lesions. Here, aiming at understanding the mechanism driven by ALK4-signalling during PDA initiation, we report the identification of ActivinA as the major ALK4-ligand expressed in pancreatic ductal neoplastic lesions. Our work pinpoints that ActivinA, which is absent in normal acinar pancreatic cells, is strongly induced in ADM-lesions promoted by Kras^{G12D} and pancreatitis in mouse and human pancreata. More importantly, we report that ActivinA induced-expression in ADMs contribute to the senescence program induced in those precursor lesions. Using ActivinA-signalling blocking-strategies, based on the injection of soluble ActRIIB-Fc molecules or genetic targeting of ALK4, we further demonstrate that ActivinA acts as a beneficial SASP factor limiting the proliferation and the expansion of pancreatic neoplastic lesions.

MATERIALS AND METHODS

Mouse Models and Experimental Procedures

All animal maintenance and experiments were performed in accordance with the animal ARRIVE guidelines and French laws and were validated by the local Animal Ethic Evaluation Committee. The generation of *Acvr1b* mutant mice has been previously described [23]. *Acvr1b*$^{flox/flox}$;*LSL-Kras*$^{G12D/+}$;*Ptf1a-Cre* (named 4KC) mice were created by combining *Acvr1b*$^{flox/flox}$ mice with the previously established *LSL-Kras*$^{G12D/+}$;*Ptf1a-Cre* (termed KC) model [24]. *Ink4a*$^{flox/flox}$;*LSL-Kras*$^{G12D/+}$;*Pdx1-Cre* mice (called KIC) had been previously described [25]. Acute pancreatitis experiments were performed using a standardized procedure [26]. Briefly, pancreatitis was induced in 1.5-month-old wild-type (WT) C57BL/6 mice using a regimen of 7 hourly intraperitoneal injections of caerulein (50 μg/kg body weight per injection; Sigma-Aldrich) dissolved in phosphate-buffered saline (PBS) for 2 consecutive days prior pancreas collection 48 hours later. Control littermates received injections of PBS only.

Immunohistological Staining and Analysis

Mouse pancreata were harvested and fixed overnight in 4% buffered formalin prior paraffin embedding and 3μm sectioning. All immuno-histological staining, unless mentioned, were performed following heat-induced epitope retrieval (antigen-unmasking solution, Vector Laboratories, UK) and primary antibodies overnight incubation at 4°C. Immunohistochemical stainings (IHC) were revealed with diaminobenzidine (DAB kit; Vector Laboratories, UK) and counterstained with haematoxylin. Immunofluorescence (IF) stainings were performed using standard protocol and counterstained with DAPI (DAPI, Vector Laboratories, UK). Phase contrast and fluorescence images were acquired on an Eclipse-NiE NIKON microscope and analysed using NIS-Elements Software. List of used primary antibodies are provided as supplemental material

(**Table S1**). BIC14011a and PA485 human tissue microarrays (TMAs) were obtained from commercial source (US Biomax, Inc.) BIC14011a is a pancreas array including grading that contains 22 pancreatitis cases, 18 pancreas intraepithelial neoplasia and 8 pancreas adenocarcinomas. PA485 is a pancreatitis and matching pancreatic adenocarcinoma array including grading and containing 43 cases of pancreatitis and matched 5 pancreatic adenocarcinomas. Sa-β-gal whole mount staining were done overnight according to the procedure from a Senescence cells histochemical Staining Kit (Sigma-Aldrich, #CS0030). Incubation and washes were all performed using fresh and filtered solutions. Collected pancreata or 3D-duct structures embedded in collagen were fixed for 10 min at room temperature using 2% paraformaldehyde/0.2% Glutaraldehyde and subsequently wash several times with PBS prior overnight incubation with Sa-β-gal staining solution in humidified sealed-chambers at 37°C. Stained tissues were subsequently washed 3 times in PBS and post-fixed with 4%-paraformaldehyde prior their embedding in Paraffin for sectioning. Combined Sa-β-gal/IF and Sa-β-gal/IHC stainings were achieved by doing IF or IHC on 7μM sections obtained from whole-mount Sa-β-Gal experiment using above-mentioned protocol. IF and Bright field images were acquired on a Zeiss Axioimager microscope and merge using ImageJ. Alcyan Blue staining were performed on rehydrated FFPE-sections using a commercial solution (Sigma-Aldrich, #B8438) and counterstaining with Nuclear Fast Red Solution (Sigma-Aldrich, #N3020).

Inhibition of Activin-signalling through soluble ActRIIB-Fc

ActivinA-signalling inhibition was achieved through the use of soluble version of the Activin type IIB Receptor (ActRIIB) coupled with a Fc domain (sActRIIB-Fc). The recombinant protein containing the ectodomain of human ActRIIB fused to the Fc domain of human IgG1 (sActRIIB-Fc) was produced as described previously [27]. For *in vivo* treatments, randomized 1.5-month-old KC mice were either injected Intraperitoneally (*i.p.*) with 5mg/kg of sActRIIB-Fc-resuspended in PBS

or PBS alone twice a week. Short and Long term treatments were achieved through 5 or 12 injections done over 3 or 6 consecutive-weeks. All pancreata were collected 24h following the last injection. For *in vitro* treatments, sActRIIB-Fc was used at a final concentration of 0.5μg/mL.

Acinar 3D Cell Culture

Primary acinar cells were isolated from mouse pancreas as previously reported [28]. Briefly, pancreata were dissected out from cervical-dislocated mice and transferred to ice-cold Hank's balanced salt solution (HBSS 1X, Life-Technologies). Pancreata were subsequently minced and dissociated with 1 mg/mL Collagenase P (Sigma-Aldrich #11213865001) in HBSS 1X for 30 minutes at 37°C. Dissociated cells were washed 3 times with HBSS 1x solution containing 10% Fetal Bovine Serum (FBS) and 10mM HEPES prior their filtration through a 100 μm sterile nylon meshes (VWR, France). Following centrifugation, pelleted cells were resuspended in 3D-culture medium (RPMI1640 medium (Gibco, #72400-021) supplemented with 25mM HEPES, 1% FBS, 1% P/S, 1μg/mL Dexamethasone and 0.1 mg/mL Soybean Trypsin Inhibitor (Sigma-Aldrich, #T6522), medium volume adjusted according to the size of pancreas [28]. 3D Culture were done in 24-well tissue culture plates coated with a 3 mg/mL layer of type I collagen from rat tail (Life Technologies, #A1048301) at least 1 h before acini isolation. Acinar isolated cells were embedded in a 1:1 mix of Rat Tail Collagen and 3D-culture medium prior their plating on the prepared collagen layers. Cell–collagen mix was allowed to solidify for 1 h at 37°C before covering the collagen discs with 1 mL of 3D culture media. Media was changed on days 1 and 3, discs and cells grown on collagen were harvested at day 6 for gene expression analysis, Sa-β-gal whole mount or paraffin embedding prior Immunohistological staining.

RNA expression analysis

RNAs were extracted using RNeasy-Kits according to manufacturer instruction (Qiagen, Valencia, USA). Real-Time PCR analyses were carried out on a Step-One RT-System (Applied-Biosystem, France). Primer-sequences are detailed in supplemental material (**Table S2**).

Statistical Analysis

All analyses were done using Prism6 software (GraphPad, USA). Statistical analyses were performed as described in figure legends; Unpaired Student t tests were used and for multiple comparisons one way Anova with Tukey post-test was used. $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$. The percentage of mice reaching endpoint was plotted using Kaplan–Meier analysis, and differences in survival were compared by log-rank test.

RESULTS

ActivinA expression is ectopically induced in acinar cells subjected to ADM promoted by pancreatitis and Kras-oncogenic transformation.

Prior exploring the role of ALK4-signalling in Kras-driven pancreatic tumorigenesis, we delineated the candidate ligands that could signal through this receptor during the onset of PDA. Taking advantage of a transcriptomic analysis performed on the *Ink4a*$^{flox/flox}$*;LSL-Kras*$^{G12D/+}$*;Pdx1-Cre* PDA-mouse model (termed KIC) [25, 29], we screened the expression of the 8 ligands reported to bind and signal through ALK4. Gene expression was analysed at 4, 6 and 9 weeks of age when KIC mice respectively develop a small number of PanINs, advanced PanINs/small pancreatic adenocarcinomas and lethal and advanced PDA [25]. Through this approach, we identified 3 candidates including *Inhba*, *Inhbb* and *Gdf11* that were showing an increased expression in the pancreas of KIC mutant-mice (**Figure 1*A***). Among the tested candidate-genes, *Gdf1, Gdf3, Gdf8, Inha and Nodal* shown a stable and moderate 1- to 1,3-fold reduced-expression in KIC samples from all ages suggesting they have a limited role in PDA onset and progression. Interestingly, while *Gdf11* and *Inhbb* were showing a moderate and rather stable expression over the time course of our analysis, *Inhba* appeared to be the most appealing candidate, showing a 5.1-, 7.4- and 17.9-fold increased expression in KIC mice (**Figure 1*A***). Such result supports that ActivinA may represent the major ALK4-signalling-ligand during pancreas tumorigenesis. We next used an immunohistological approach to detect ActivinA within pancreatic lesion from different grades. While ActivinA was not expressed in Acinar-, islet- and Ck19-positive duct-cells within normal pancreata, it was detected in all KIC-analysed lesions ranging from ADM to Adenocarcinoma (**Figure 1*B***). Colocalization experiments revealed that ActivinA expression was the most prominent and homogenous throughout ADM-lesions following the commitment of acinar-cells into Ck19-duct expressing cells (**Figure 1*C*)**. Identification of a reduced-number of cells expressing ActivinA in the lining of PanINs1/2 and the diffuse expression of ActivinA in PanIN3 and

Adenocarcinoma lesions (**Figure 1*C***) led us to speculate that the ectopic induction of ActivinA in ADM may represents an acute response to the oncogenic insult driven by the combined ablation of p16 and Kras activation. We next questioned whether ActivinA induced-expression was also taking place in ADM-promoted by other pathological contexts such as the sole activation of *Kras*^{G12D} in acinar cells or the inflammatory cues that drives pancreatitis. Using *LSL-Kras*$^{G12D/+}$;*Ptf1a-Cre* (termed KC) and caerulein-injection in WT animals as respective models of low-graded neoplastic lesions [30] and pancreatitis [31] we found that ActivinA was strongly induced in acinar-to-duct converting cells independently of the triggering signal driving ADM (**Figure 2*A* and 2*B***). Interestingly, as found in KIC pancreas, we confirmed that following its induction in ADM, ActivinA expression was only maintained in a subset of cells lining within PanINs lesions in KC mice (**Figure 2*A***). Finally, using a human pancreatic tissue microarrays we further validated the lack of ActivinA expression in normal acinar cells and the existence of a robust ActivinA expression in 60% of ADM-lesions seen in patients developing pancreatitis (n=64) whereas ActivinA immunoreactivity was reduced and limited to a subset of duct-cells in low-graded PanINs (**Figure 2*C***).

Targeting ActivinA in KC mice accelerates the formation of ADM/PanIN lesions

Having found ActivinA to be ectopically expressed in acinar cells during ADM, we next addressed the consequences of blocking its signalling. We first used sActRIIB-Fc, a soluble Activin receptor, to inhibit ActivinA signalling through ALK4 [27]. Short treatments (ST) based on 5 intra-peritoneal (i.p.) injections of sActRIIB-Fc performed over a 3-week time-period was increasing significantly pancreas-weights of 1.5-month-old treated KC mice compared to aged-matched WT and non-treated KC controls (**Figure 3A, B**). Histological analysis of treated-KC animal revealed that the use of sActRIIB-Fc was accelerating the development of ADM- and PanIN-lesions as addressed by their surface measurements (**Figure 3*C***). These observations were subsequently confirmed

through the use of an ALK4 conditional KO mouse model we previously generated [23]. Following the generation of *Acvr1b*$^{flox/flox}$;*LSL-Kras*$^{G12D/+}$;*Ptf1a-Cre* compound-mutant mice (termed 4KC) and the histological analysis of their pancreata, we confirmed that targeting ALK4 in mice harbouring *Kras*^{G12D} mutations was driving an accelerated development of IPMNs as previously reported using a *Pdx1-Cre* diver line [22] (Data not shown). More interestingly, while the analysis of 3-week-old pancreata from KC and 4KC animals did not reveal obvious ADM or PanIN lesions, we found the pancreas of 1.5-month-aged 4KC mice to be enlarged and presenting an increased number of ADM lesions (**Figure 3*D*, S1**). Accelerated ADM formation in 4KC pancreata was further confirmed by the identification of an increased Ck19 immunoreactivity, a more pronounced mucin and collagen deposition and a larger number of Desmin+ pancreatic-stellate cells recruited within the developed lesions (**Figure 3*C* and S1*C***).

ADM-lesions associate with a senescence signature that colocalizes with ActivinA expression

Gene-expression screenings of cells undergoing OIS have pointed *Inhba* as a SASP-candidate [19]. Knowing that *Inhba* is also a target of Kras oncogenic activation in pancreatic duct cell [20], we hypothesize that the induced-expression of ActivinA identified in ADM may underline a Kras-OIS in those lesions. Kras^{G12D} mutated acinar-cells grown in 3D-collagen matrix form cystic-duct structures that resemble and recapitulate ADM [28]. Using such model, we found *Inhba* expression to be induced during the formation of duct-structures generated from 6-day-cultured KC-acinar cells (**Figure 4*A***). While the analysis of those differentiated structures confirmed the loss of acinar markers and the increase of duct-genes and proteins expression (**Figure S2**), we found them to contain senescent cells as addressed by the use of a Senescence-Associated Beta-Galactosidase assay (Sa-β-Gal) (**Figure 4*B***). Senescence was further confirmed through a significant increased-expression of the putative *Cdkn1a* (p21), *Cdkn2a* (p16) senescence-associated cyclins kinase inhibitor and a number of SASP-associated genes such as *Il6*, *Il1a*, *Ccl20* and *Vegfc* (**Figure**

4*C*). Having demonstrated that acinar derived duct-structures were subjected to senescence and shown an *InhbA* induced-expression, we next addressed whether ActivinA was produced by senescent cells during ADM. Using pancreata from KC mice we first confirmed the induction of a senescence-phenotype in ADM and PanINs lesions as previously pointed (**Figure 4*D***) [12]. Interestingly, we noted that the patterns of Sa-β-Gal+ cells detected in both ADM and PanINs-lesions were reminiscent of the expression pattern of ActivinA+ found in KIC, KC and caerulein-induced lesions (**Figure 1C, 2 and 4*D***). Colocalization of ActivinA expression in Sa-β-Gal+ cells was confirmed so the expression of p21 and γH2AX senescence markers in ADM lesions expressing ActivinA (**Figure 4*E* and 4*F***). Taken together, those results support the role of ActivinA as a SASP component in ADM-lesions and they further point the potent importance of ActivinA-signalling through ALK4 within the senescence program that is promoted in those lesions.

Inhibition of ActivinA-signalling reduce Oncogene-Induced Senescence in ADM

Having found that targeting ActivinA-signalling drives an accelerated ADM formation, we next addressed whether such observation reflects an escape to the senescence program promoted by *Kras*^{G12D} activation. Treatment of KC-derived acinar-cells with sActRIIB-Fc confirmed that duct-structures obtained from 6-days treated-cells were less prone to senescence as demonstrated by the reduced Sa-β-Gal staining and the decreased expression of the senescence-associated cyclin-dependent kinase Inhibitor *Cdkn1a* and *Cdkn2a* (**Figure5*A*, 5*B* and S3*A***). Surprisingly, inhibition of ActivinA-signalling was showing a moderate impact on the regulation on tested SASP-candidates and while the expression of *Ccl20* and *Vegfc* was significantly reduced upon sActRIIB-Fc treatments, the expression of *Il6*, *Il1a*, *Il1b* or *Ccl2* was not impacted (**Figure 5*B***). Histological analysis of paraffin embedded structures did confirm that yet senescence was reduced in the formed cysts, the differentiation of ADM-structures was not

impacted following the inhibition of ActivinA-signalling as assessed by the evaluation of Sox9 and Ck19 duct-markers expression (**Figure 5*C* and S3*B***). Finally, we found that the decreased senescence identified through Sa-β-Gal assays was further supported by a robust and significant reduction of p21 and γH2AX expression among the Ck19+ cells within duct-structures formed from KC-sActRIIB-Fc treated and 4KC acinar cells (**Figure 5*D*, 5*E* and S3*C***). Such observation supports that blocking ActivinA-signalling through ALK4 does not impact the formation of ADM but rather impacts the mechanism driving senescence escape among pre-neoplastic ADM-cells. We further explored this hypothesis in 4KC and in KC animals exposed to short sActRIIB-Fc treatment. As shown in **Figure 5*F* and S4*A***, we found that blocking ActivinA-signalling was resulting in a reduced Sa-β-Gal activity within ADM and PanINs lesions from both models. Reduced observed-senescence was associated with a significant decreased-expression of p21 and γH2AX in pancreas areas that were presenting ADMs (**Figure 5*G, H* and S4*B, C***). This result supports that, *in vivo*, ActivinA-signalling is contributing to the senescence cues seen in Kras-mediated ADM and PanINs.

Senescence escape mediated by ActivinA-signalling inhibition results in the formation of proliferative lesions

Senescence bypass is a key feature of tumour initiation and progression and it is well-accepted that OIS leads to a transcriptional repression of proliferation through activation of the p53-p21 and/or p16-Rb suppressor pathways [7]. Having found that blocking ActivinA-signalling results in a reduced senescence and a decreased expression of the cell cycle inhibitor p21, we next thought to determine whether those observations were associated with a promoted-proliferation. Interestingly, duct-like structures formed from sActRIIB-Fc treated and 4KC-isolated cells were frequently showing enlarged-size compared to KC-untreated and KC-age-matched controls. Quantification of paraffin-embedded structures confirmed that the chemical and genetic

inhibition of ActivinA-signalling was driving the formation of cysts with large diameters (**Figure 6*A* and S5*A***). Using Ki67 as a proliferative marker, we found that those structures cells were presenting an increased percentage of Ki67 positive cells per duct compared to their matching controls (**Figure 6*B* and S5*B***). Consistent with these observation, analysis of the pancreata of 4KC and sActRIIB-Fc treated KC mice further confirmed that ADM lesions were presenting a significant increased proliferation compared to age-matched control pancreata (**Figure 6*C* and S5*C***). Interestingly, we found that the proliferation was sustained in PanINs lesions following sActRIIB-Fc treatment highlighting that inhibition of ActivinA-signalling may favour the formation of aggressive lesions with increased proliferative potential (**Figure 6*C***).

Senescence-escape mediated by inhibition of ActivinA-signalling drives an accelerated progression of pancreatic tumours and the formation of cystic lesions in vivo

We next questioned the consequences of a sustained ActivinA-inhibition on *Kras*^{G12D}-acinar cell transformation. Long term analysis of 4KC mice highlighted that disrupting ALK4 expression dramatically reduces the survival of KC animals pointing that less than 50% of the animals are surviving 7-months-of-age (**Figure S6*A***). This result is consistent with the reduced survival seen in patients presenting a reduced *ACVR1B* expression (data not shown). Analysis of 14-week and 30-week-old 4KC mice confirmed the accelerated progression of ADM to PanINs-graded lesions within the entire pancreas (**Figure 7*A*** and Data Not shown). 4KC mutant animal were found to develop Ck19+ cystic lesions lacking ALK4 that were reminiscent of the IPMNs reported within the *Acvr1b*$^{flox/flox}$;*LSL-Kras*$^{G12D/+}$;*Pdx1-Cre* model developed by Qiu *et al.* [22] (**Figure 7*A*, 7*B*, S6**). Subsequent analysis revealed that 4KC pancreata were presenting a large number of proliferating Ki67+/Ck19+ cells adjacent to each-others and located within the lining of the cystic-lesions (**Figure 7*C***). To further validate these observations, we thought to explore the consequences of long term sActRIIB-Fc treatment on KC mice. To that extent, 1.5-month-old KC mice were

IP-injected twice a week during 6 weeks (LT Treatment) prior the histological analysis of their pancreas. Consistent with our finding in 4KC-aged mice, analysis of LT treated KC mice confirmed that the inhibition of ActivinA-signalling, hence the senescence escape it promotes in ADM, was leading to an accelerated formation of both PanINs and Ck19+ cystic lesions that could be found within the pancreata of sActRIIB-Fc treated mice (**Figure 7*D* and 7*E***). As found within the 4KC cystic-lesions, we observed a significant increased-percentage of proliferating Ck19+ cells that were frequently adjacent and located in the lining of the cystic-formed lesions (**Figure 7*F***).

DISCUSSION

Contribution of ALK4 in the onset of pancreatic cancer have been highlighted through the identification of *ACVR1B* mutations in PDA and use of ALK4-KO mouse models [22, 32, 33]. While the role of ALK4-signalling had been clearly demonstrated in driving the aggressiveness of pancreas cancers and the formation of IPMNs [22, 32, 33], little was known about the ligands involved in the mechanisms taking place through ALK4 during pancreatic tumour initiation. Here, we report ActivinA to be the most prominent ALK4 ligands-expressed during the onset of mouse pancreatic tumours. We show ActivinA expression to be strongly induced in ADM lesions, and subsequently limited to a subset of cells while lesions are progressing to PanIN grades in both mouse and human. Our observations point ActivinA as a major SASP factor in the OIS promoted by Kras within ADM. They further support that ActivinA production by neoplastic-senescent-cells block the proliferation of ADM and hence limit their progression into PanINs and more advanced lesions through the modulation of p16 or p21 expression. Beside those observations, our work also pinpoints the importance of Kras-OIS in limiting the progression of ADMs to more advanced lesions and further demonstrates that a SASP-factor could exert a beneficial anti-tumoral role. These results emphasize that blocking ActivinA-signalling through ALK4 reduces senescence *in vitro* and *in vivo*, leading to the increased proliferation of ADM- and PanIN-lesions, an observations consistent with the reduced survival of patients presenting ALK4 mutations [33].

The implication of ActivinA in PDA is supported by clinical studies reporting its expression in a subset of human pancreatic cancers and the identification of elevated ActivinA blood-levels in PDA-patients [34, 35]. ActivinA had been reported to drive the self-renewal of pancreatic cancer-stem cells [36, 37] and to promote pancreatic cancer-associated cachexia [35, 38]. Our results underline that beside these pro-tumoral functions in advanced lesions, ActivinA-signalling play a suppressive role in the early stage of pancreatic tumour initiation through its SASP-associated function. This

observation is important as it reinforce the existence of ant-tumoral SASP-molecules such ActivinA that exert beneficial effect *in vivo* during OIS.

While members of the TGFβ-signalling family have been associated with a pro-fibrotic senescence phenotype [39] in opposition to the pro-inflammatory SASP mediated by NF-κB, ligands of the TGFβ-superfamily have been further proposed to be important actors of the paracrine senescence mediated by Kras [19]. Blockade of TGFβ-signalling have shown to respectively enhance oncogenic Ras-induced tumorigenesis and metastasis in mammary epithelial cells and reduced senescence in pancreatic cancer model [19, 40]. Proteomic analysis has unveiled the dynamic pattern of Ras-related SASP, pointing the role of TGFβ-signalling in the early stage of the senescence process [39] and identifying INHBA, GDF15, BMP2 and BMP6 as the major actors of the paracrine SASP, while TGFβ1 was moderately induced compared to the above-mentioned ligands [19]. These observations highlight that ActivinA may represent one of the key element of the TGFβ-signalling paracrine senescence, a hypothesis supported by the proposed use of ActivinA a potential blood biomarker for senescent cell burden in vivo [41] and consistent with the recent published role of ActivinA as a prominent autocrine regulator of hepatocytes growth arrest and cellular senescence through *CDKN2B*/*p15*^{ink4a} [42].

Whereas ActivinA and its signalling through ALK4 limit ADM/PanIN progression, cell- and non-cell-autonomous molecular targets downstream of this signalling remain to be further defined. Previous work has pointed ActivinA-capable of inducing p21 through Smad-signalling [43]. Interestingly, p21 has been reported to limit senescence and ADM formation during pancreatitis [44]. To the same extent, the suppression of p21 growth arrest accelerates the progression of Kras pancreatic lesions through an increased proliferation [45]. Taken together these observations present p21 as a plausible target of ActivinA signalling in epithelial cells, a result supported by the combined reduced p21 expression/increased proliferation found in ADM/PanIN lesions following Activin-signalling inhibition.

From a general perspective, our data underlines that a large population of cells undergoing ADM express ActivinA and are committed to a senescent phenotype, while only a limited number of senescent cells can be detected in PanINs. Based on this observation, it is important to define the origin of the cells that remains senescent through their progression into PanINs and whether they exert a function as senescence driver-cells capable of modulating the various arms of the pro-inflammatory and fibrotic SASP or through the more recently identified senescence-associated stemness (SAS) [46].

Finally, one may question the relevance of our work for the clinics. Targeting ActivinA through the use of anti-ActRIIB blockade has been proposed as an effective intervention against muscle atrophic-conditions and cancer cachexia and more recently senescence-associated aging conditions [47]. Promising therapeutic benefits have been shown on reverting cancer-cachexia promoted by ActivinA or Myostatin production by ovarian tumour and xenografted lung and colon cancer cell lines [48-50]. Our results pinpoint that the use of such therapy, while efficient in many pathological-conditions, should be considered with extreme caution in the case of pancreatic cancer. Indeed, knowing that low grade pancreatic lesions are frequently found in peri-tumoral regions surrounding PDA, one may question whether the benefit of blocking ActivinA-induced cachexia through the use of sActRIIB-Fc strategy would favour the progression of neoplastic lesions into more advanced grades.

In conclusion, our work provides the first demonstration that ActivinA and its signalling through ALK4 represent one of the first defence that is induced through Kras-OIS to limit the development and progression of PanIN. While limited to the sole function of ActivinA as an anti-tumoral SASP-factor our work emphasizes the need to better comprehend the complex actions of the whole SASP molecules that will be produced by ADM lesions and could take an important anti- or pro-tumorigenic role in both pre-neoplastic cells and their cellular environment. It is thus evident that understanding the impact of senescence cells and their SASP within low grade pancreatic

neoplasm should represents a key step to develop innovative therapeutic strategies for pancreatic cancer.

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FIGURE LEGENDS

Figure 1: Expression of ActivinA is induced during the onset of pancreatic tumour developed by KIC mice. (A) Gene-expression analysis of ALK4-ligands performed of a transciptomic data-set obtain from *Ink4a*$^{flox/flox}$;*LSL-Kras*$^{G12D/+}$;*Pdx1-Cre* (KIC) pancreata. Fold of relative gene expression is shown at indicated-age when samples were collected from control and KIC mice. ***(B)*** Immunohistochemical staining of ActivinA performed on WT of KIC FFPE-pancreas sections, right column shows enlarged views of the dashed areas. PanIN and Adenocarcinoma grades were stained on sections obtain from 4 and 9-week-old KIC mice respectively. ***(C)*** Representative pictures of Ck19 and ActivinA double IFs performed on WT of KIC FFPE-pancreas sections. Right columns are enlarged views of shown pictures. Pancreatic islets (isl) are dashed in WT control. Grades and scale bars are indicated.

Figure 2: ActivinA is expressed in ADM-lesions induced during pancreatitis and by Kras^{G12D} oncogenic transformation. ***(A, B)*** Representative pictures of Ck19 and ActivinA double IFs done on FFPE-pancreas sections obtained from ages-match WT, *LSL-Kras*$^{G12D/+}$;*Ptf1a-Cre* (KC) or Caerulein-treated WT mice. Right column shows enlarged views of the dashed areas. Grades and scale bars are indicated. ***(A)*** WT and KC mice were analysed at 1.5 months of age. ***(B)*** IFs were performed on pancreas collected 48h after 2 consecutive days of 7 hourly Caerulein or PBS i.p. injections. ***(C)*** Immunohistochemical analysis of ActivinA expression on human pancreas tissue microarrays, 2 independent samples are shown for each grades shown. Insets show enlarged-view of the dashed area. Quantifications of the ActivinA immunoreactivity are shown for the Pancreatitis (n=65) and PanIN1/2 (n=18) samples present from 2 independent TMAs (BIC14011a and PA485,US Biomax, Inc.).

Figure 3: In vivo Inhibition of ActivinA-Signalling favours ADM formation. (A) Schematic skim depicting the experimental design of Short-Term (ST) sActRIIB-Fc treatments. 5mg/kg of sActRIIB-Fc or matching volume of PBS (vehicle) were *i.p.* injected to 1.5-month-old KC animals twice a week (n=5 animals/group). ***(B)*** Representative pictures of pancreas collected following ST treatment, weights of sActRIIB-Fc or Vehicle treated pancreata are indicated, a representative WT pancreas is show as reference. Right panel shows a quantification of pancreas weights (n=5 per group). ***(C)*** Representative Haematoxylin & Eosin (H&E) colorations of KC pancreas collected from sActRIIB-Fc or vehicle treated mice. ADM/PanIN lesions are circled by dashed-lines and quantification are shown for each individual in the histogram. ***(D)*** Representative H&E colorations of age-matched *LSL-Kras*$^{G12D/+}$;*Ptf1a-Cre* (KC) and *Acvr1b*$^{flox/flox}$;*LSL-Kras*$^{G12D/+}$;*Ptf1a-Cre* (4KC) mice. Pictures of 3-week-old and 1.5-month-old pancreata are shown. Further characterisation of ADM is highlighted through Ck19 IHCs and Alcyan-blue staining provided in the right panel. Dashed-lines underline the lesions seen in the Alcyan-blue pictures. A surface quantification of ADM lesions performed on 1.5-month-old KC (n=6) and 4KC (n=9) mice is shown. Means ± SEM are shown. $^{*}P < 0.05$; $^{***}P < 0.005$; Student *t* test.

Figure 4: ActivinA is expressed in ADM cells undergoing senescence. (A) Day 1 and day 6 bright field images of KC isolated acinar-cells grown in 3D-rat collagen. Note the duct-like structure that are formed at day 6. Quantification of *InhbA* gene expression by real-time quantitative PCR analysed at day1 and day6 (4 independent experiments were done). ***(B)*** Representative FFPE-section of a whole-mount Sa-β-Gal staining performed on acinar-cell derived duct-structures from a 1.5-month-old KC pancreas cultured in 3D-collagen for 6 days***.*** Arrowheads point senescent cells positive for Sa-β-Gal. ***(C)*** Quantitative reverse transcription polymerase chain reaction (RT-PCR) of the indicated Senescence- (*Cdkn1a, Cdkn2a*) and SASP-associated (*Il6, Il1a, Il1b, Ccl2, Ccl20. Vegfc*) genes. Acinar-cell culture from 1.5-month-old KC mice were analysed at

indicated time points. Means ± SEM are shown. $^{*}P < 0.05$; $^{***}P < 0.005$; *ns: non-significant,* Student *t* test. ***(D)*** FFPE-section of whole-mount Sa-β-Gal staining performed on 1.5-month-old WT and KC pancreas ADM and PanINs lesions are indicated by arrows or delineated by dashed lines (left panel). Representative Sa-β-Gal staining combined with Ck19 IHCs are shown in right panel to highlight the preponderant senescence in ADM and the weak expression of Ck19 in senescent cells seen in PanINs. Lower row represents magnified views of the dashed-squares. 1.5-months old KC mice were analysed. ***(E)*** Colocalization of Sa-β-Gal reactive areas with ActivinA expression in section of ADM from 1.5-month-old KC mice. Merged ActivinA-IF and Sa-β-Gal bright-field images taken from the same stained section are shown magnified on the right. ***(F)*** IHCs of ActivinA and indicated Acinar (Amylase), ADM/Duct (Sox9) and senescence (γH2AX, p21) markers are shown in ADM Sections of KC mice or normal WT pancreas. Scale bars are indicated.

Figure 5: Inhibition of ActivinA-signalling mediated by sActRIIB-Fc reduces Kras-OIS in ADM. (A) Representative FFPE-section of a whole-mount Sa-β-Gal staining done on KC acinar-cells cultured in 3D-collagen for 6 days with sActRIIB-Fc (KC + sActRIIB-Fc) or vehicle (KC)**.** Note that all experiments were performed with KC cells isolated from 1.5-month-old animals, and that sActRIIB-Fc treated and non-treated cells are from the same animal in each individual experiment (n=4). Quantification of the percentage of Sa-β-Gal positive cells per fields is shown. ***(B)*** Quantitative RT-PCR of the indicated Senescence and SASP genes. Acinar-cell culture from 1.5-month-old KC mice grown in differentiation media supplemented with sActRIIB-Fc or PBS were analysed after 6 days of culture. ***(C-E)*** Representative double IF staining for Ck19/Sox9 *(C)*, Ck19/p21 *(D)* and Ck19/γH2AX *(E)* performed on FFPE-embedded duct-structures obtained from KC acinar-cells cultured in 3D-collagen for 6 days with sActRIIB-Fc or Vehicle. Insets show magnified view of stained-cells within formed duct-structures. Quantification of relative Sox9

expression *(C)* and percentage of p21 and γH2AH positive cell *(D, E)* are shown for the 5 independent experiments performed. ***(F)*** FFPE-section of whole-mount Sa-β-Gal staining performed on the pancreas of 1.5-month-old KC mice exposed to a short sActRIIB-Fc or vehicle treatment (ST). Representative pictures of Sa-β-Gal reactivity in PanIN and ADM lesions and quantification of ADM/PanIN surfaces positive for Sa-β-gal are shown (experiments were performed on groups of 5 mice). ***(G,H)*** Pictures of p21 and γH2AX IHCs done on ADM sections obtained from KC mice following ST treatment with sActRIIB-Fc or vehicle. Quantification are shown in right panels (n=5 animals/group) Scale bars are indicated. Means ± SEM are shown. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; *ns: non-significant,* Student *t* test.

Figure 6: Inhibition of ActivinA-signalling promotes cell proliferation in ADM. (A, B) IHC and IF stainings performed on 3μM sections of FFPE-embedded duct-structures obtained from KC acinar-cells cultured in 3D-collagen for 6 days with sActRIIB-Fc or vehicle. Right column show magnified view of dashed squares. ***(A)*** Representative Ck19 IHC staining showing the enlarged diameters of duct-structures formed from sActRIIB-fc-treated KC acinar-cells. Quantification are shown. ***(B)*** Representative Ck19/Ki67 IF highlighting the increased proliferation seen in sActRIIB-Fc-treated cells. Insets show magnified view of stained cells. Quantification of Ki67% cells per individual duct-structures are provided (n=21 to 27 structures from 3 independent experiment were analysed). ***(C)*** Representative pictures of Ck19/Ki67 double IFs done on ADM and PanIN sections obtained from KC mice following ST treatment with sActRIIB-Fc or Vehicle. Quantification are shown in right panels (n=5 animals/group). Scale bars are indicated. Means ± SEM are shown. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; *ns: non-significant,* Student *t* test.

Figure 7: Targeting ActivinA-Signalling accelerates the progression of ADM into proliferative Ck19+ cystic lesions. (A) Representative H&E colorations of pancreata collected from 14- and 30-week-old KC and 4KC mice. ***(B)*** Macroscopic picture of a 30-week-old 4KC pancreas with cystic lesions (black arrowheads). A magnified view of the cystic lesion pointed by the white arrowhead is shown. A Ck19 IHC of the shown pancreas underline the cystic nature of formed Ck19 ductal lesions. ***(C)*** Representative Ck19/Ki67 double IF of cystic lesions detected in the pancreata of 3 independent 30-week-old 4KC mice***.*** Lower panels are magnified views of the dashed-rectangles showing Ki67-proliferative cells lining in stripes within the wall of the pancreatic cystic lesions. ***(D)*** Experimental design of the Long-Term (LT) sActRIIB-Fc treatments and H&E staining of representative cystic lesions that develop in KC sActrIIB-Fc LT-treated mice compared to vehicle treated ones. Quantification of ADM/PanIN and cystic lesions are shown (n=3 mice were analysed for each condition) ***(E)*** Weights of LT-treated pancreata are indicated for sActRIIB-Fc (n=3) or Vehicle groups (n=3), representative pancreas are show. ***(F)*** Representative Ck19/Ki67 double IF of the lesions found in the pancreas of LT-treated KC mice***.*** Lower panels are magnified views of dashed-squares; quantification of Ki67 proliferative Ck19+ cells is shown (n=3 mice analysed/group). Note that both cystic and non-cystic lesions are demonstrating an increased proliferative capacity. Scale bars are indicated. Means ± SEM are shown. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; *ns: non-significant,* Student *t* test.

Supplemetal Material & Method

Sirius-Red histological coloration.

Tissue slides were dewaxed, rehydrated, and stained with Picro-sirius red (Sigma-Aldrich, #365548) for 1 hour. Stained slides were then washed in two changes of acidified water and dipped into 95%-100%-100% ethanol bathes for dehydration. Slides were finally cleared in xylene prior their mounting in Pertex.

Supplemental Figure Legends

Supplemental Figure 1: **Inactivation of *Acvr1b* in the context of oncogenic Kras^{G12D} results in the development of enlarged ADM lesions.** ***(A)*** Weights of 1.5 to 2.5-month-old WT (n=20), KC (n=24) and 4KC (n=13) pancreata. ***(B)*** Quantification of the number of individual ADM lesions found in KC (n=6) and 4KC (n=9) mice at 1.5 months of age. ***(C)*** Representative pictures of H&E, Desmin Immuno-staining and Sirius-Red collagen-coloration in the pancreas of 1.5-month-old KC and 4KC mice. Dashed-lines identify individual ADM lesion as counted in *(B)*. Means ± SEM are shown. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$, Student *t* test.

Supplemetal Figure 2: Cultures of KC-acinar cells embeded in 3D-collagen recapitulate ADM. (A, B) Quantitative reverse transcription polymerase chain reaction (RT-PCR) of the indicated Acinar- (*Amy2a, Rbpjl, Cpa1, Ptf1a, Cela1*) and Duct-associated (*Sox9, Rbpj, Pdx1, Hes1, Ck7*) genes. Acinar collagen-cell-culture from 1.5-month-old KC mice were analysed at indicated time points. Results were obtained from independent cultures done from 4 independent isolation of KC-acinar cells. ***(C)*** Representative IHC staining for Ck19 and Sox9 performed on 3mM-thick histological sections of FFPE-embedded duct-structures

obtained from KC acinar-cells cultured for 6 days in 3D-collagen. Arrowheads point Sox9 positive nuclei. Means ± SEM are shown. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$, Student *t* test.

Supplemental Figure 3: Disruption of ALK4-expression reduces Kras-OIS in ADM in vitro. ***(A)*** Representative FFPE 3mM-section of a whole-mount Sa-b-Gal staining done on KC and 4KC acinar-cells cultured in 3D-collagen for 6 days***.*** Note that all experiments were performed with Acinar cells isolated from 1 to 1.5-month-old animals. Quantification of the percentage of Sa-b-Gal positive cells per fields is shown (KC n=3, 4KC n=3). ***(B, C)*** Representative double IF staining for Ck19/Sox9 *(B)*, Ck19/p21 and Ck19/gH2AX *(C)* performed on FFPE-embedded duct-structures obtained from KC and 4KC acinar-cells cultured in 3D-collagen for 6 days. Quantification of the % of Sox9, p21 and gH2AX positive cells per duct are shown (B, C). 16 and 14 independent duct structures were respectively evaluated from independent KC and 4KC-acinar cell culture. Means ± SEM are shown. $^{*}P < 0.05$; $^{***}P < 0.005$; *ns: non-significant,* Student *t* test.

Supplemental Figure 4: Disruption of ALK4-expression reduces Kras-OIS in ADM in vivo. ***(A)*** FFPE-section of whole-mount Sa-b-Gal staining performed on the pancreas of 1.5-month-old KC and 4KC mice. Representative pictures of Sa-b-Gal reactivity in PanIN lesions and quantification of ADM/PanIN surfaces positive for Sa-b-gal are shown (experiments were performed on n=9 KC and n=7 4KC animals). ***(B,C)*** Pictures of p21 and gH2AX IHCs done on ADM sections obtained from 1.5-month-old KC and 4KC mice. Quantification are shown in right panels (n=4 animals/group), arrowheads point p21-positive cells that were counted. Means ± SEM are shown. $^{**}P < 0.01$; $^{***}P < 0.005$, Student *t* test.

Supplemental Figure 5: Disruption of ALK4-expression promotes proliferation of ADM lesions. (A, B) IHC and IF stainings performed on 3mM sections of FFPE-embedded duct-structures obtained from KC and 4KC acinar-cells cultured in 3D-collagen for 6 days. Note that all experiments were performed with Acinar cells isolated from 1 to 1.5-month-old animals ***(A)*** Representative Ck19 IHC staining showing the enlarged diameters of duct-structures formed from 4KC acinar-cell compared to KC-cultures. Quantification are shown. ***(B)*** Representative Ck19/Ki67 IF highlighting the increased proliferation seen in 4KC cells. Quantification of Ki67% cells per individual duct-structures are provided (n=3 (KC), n=10 (4KC)). ***(C)*** Representative pictures of Ck19/Ki67 double IFs done on ADM sections obtained from 1.5-month-old KC and 4KC mice. Quantification are shown in right panels (n=3 animals/group). Scale bars are indicated. Means ± SEM are shown. $^{**}P < 0.01$; $^{***}P < 0.005$, Student *t* test.

Supplemental Figure 6: Disruption of ALK4-expression promotes the formation of pancreatic cystic lesions. (A) Macroscopic picture of a 30-week-old age-matched WT, KC and 4KC pancreas. Survival curves of KC and 4KC mice are shown. ***(B)*** Immunohistochemical staining of ALK4 performed on KC and 4KC FFPE-pancreas sections. Loss of Alk4 expression is shown in PanINs and Pancreatic cystic lesions developped by 4KC animals. ***(C)*** Representative pictures of Alcyan-blue coloration done on FFPE-pancreas-sections of age-matched KC and 4KC animals $^{***}P < 0.005$; the percentage

of mice reaching endpoint was plotted using Kaplan–Meier software, and differences in survival were compared by log-rank test.

Supplemental Table 1. List of used Primary antibodies.

Antigen	***Species***	***Source***	***Dilution***	
			IHC	IF
ALK4	Rabbit	Abcam (ab109300)	1:5000	/
Amylase	Rabbit	Sigma (A8273)	1:5000	/
Cytokeratin19	Rat	DHSB (Troma-III)	1:100	1:20
Desmin	Goat	R&D Systems (AF3844)	1:2500	/
Phospho-H2AX (gH2AX-ser139)	Rabbit	Cell Signalling (#9718)	1:1500	1:300
ActivinA	Rabbit	Genetex Inc. (GTX108405)	1:500	1:1000*
Ki67	Goat	SCBT (sc-7846)	1:750	1:750*
p21WAF1/Cip1	Mouse	Dako (M7202)	1:100	1:50
Sox9	Rabbit	Sigma (C89924)	1:5000	1:100

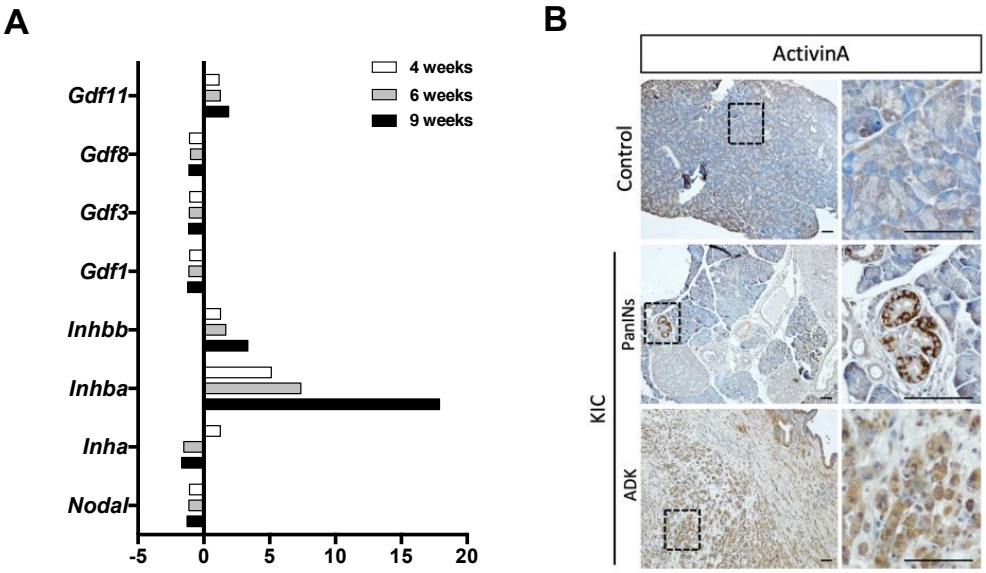
* Combined with a Tyramide Signal Amplification (TSA system, Perkin-Elmer)

Supplemental Table 2. Primer pairs used for real-time quantitative RT-PCR.

Gene name	**Forward primer sequence (5'-3')**	**Reverse primer sequence (5'-3')**
InhBA	CCAAGCTGAGACCCATGTCC	CCACTGTCTTCTCTGGACTCTC
Cdkn1a	GCAGATCCACAGCGATATCC	CAACTGCTCACTGTCCACGG
Cdkn2a	CCCAACGCCCCGAACT	GCAGAAGAGCTGCTACGTGAA
IL6	TGATTGTATGAACAACGATGATGC	GGACTCTGGCTTTGTCTTTCTTGT
IL1a	CGCTTGAGTCGGCAAAGAAAT	TGGCAGAACTGTAGTCTTCGT
IL1b	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
Ccl2	CATCCACGTGTTGGCTCA	GATCATCTTGCTGGTGAATGAGT
Ccl20	ACTGGGTGAAAAGGGCTGT	GTCCAATTCCATCCCAAAAA
Vegfc	CCAGCACAGGTTACCTCAGCAA	TAGACATGCACCGGCACCAA
Amy2a	TGCAGGTCTCTCCACCCAATGAAA	TGCACCTTGTCACCATGTCTCTGA
Rbpjl	ATGCCAAGGTGGCTCAGAAAT	CTTGGTCTTGCATTGGCTTCA
Cpa1	TACACCCACAAAACGAATCGC	GCCACGGTAAGTTTCTGAGCA
Ptf1a	TGCGCTTGGCCATAGGCTACATTA	AGATGATAACCTTCTGGGCCTGGT
Cela1	AATGTCATTGCCTCCAACTGAGCG	ATTAGACAAGTGCTCGGCCACTGA
Sox9	CAAGACTCTGGGCAAGCTCTG	TCCGCTTGTCCGTTCTTCAC
Rbpj	GCTGACTTGTGCATTGCTTCAGGA	CCACTGTTGTGAACTGGCGTGGAA
Pdx1	AAATCCACCAAAGCTCACGC	CGGTCAAGTTCAACATCACTGC
Hes1	AGAGAAGGCAGACATTCTGGA	GTCACCTCGTTCATGCACTC
Krt7	CACGAACAAGGTGGAGTTGGA	TGTCTGAGATCTGCGACTGCA
*GAPDH**	CAACGACCCCTTCATTGACC	GGTCTCGCTCCTGGAAGATG
*HPRT**	GTTGGGCTTACCTCACTGCT	TAATCACGACGCTGGGACTG

*: genes used as housekeeping references.

Figure 1, Zhao *et al.*



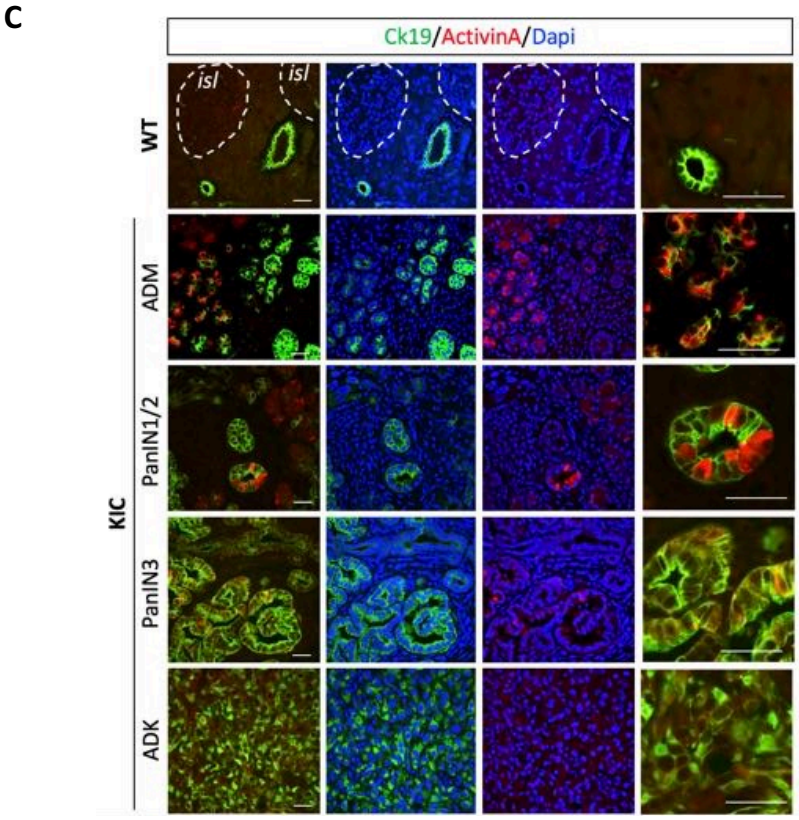


Figure 2, Zhao *et al.*

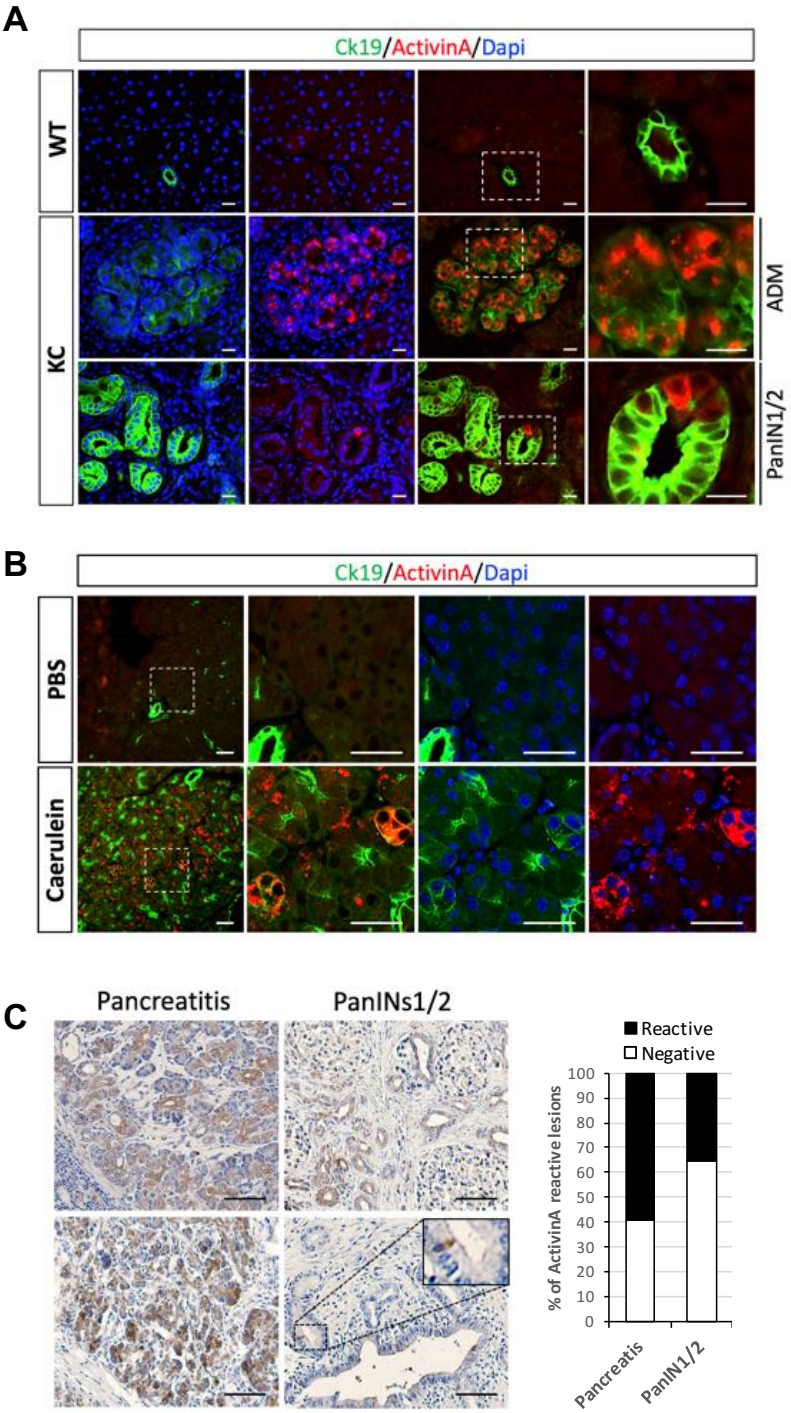


Figure 3, Zhao *et al.*

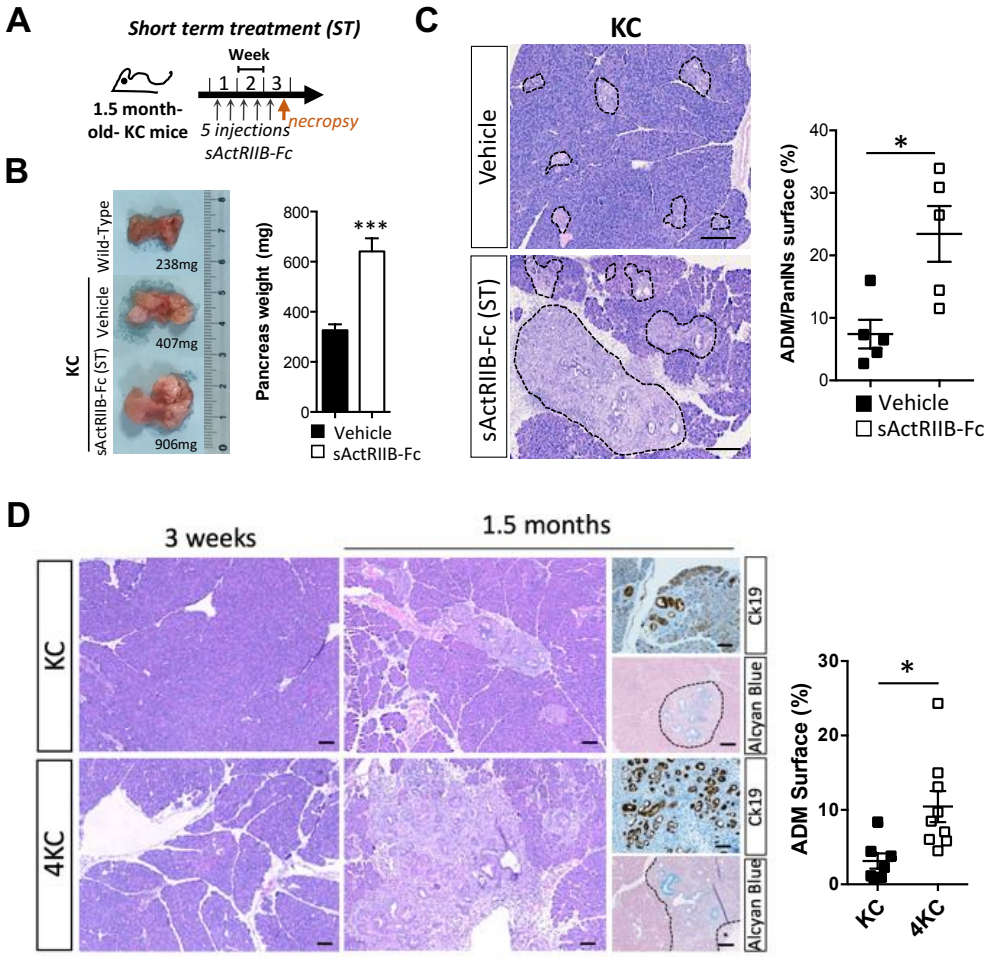


Figure 4, Zhao *et al.*

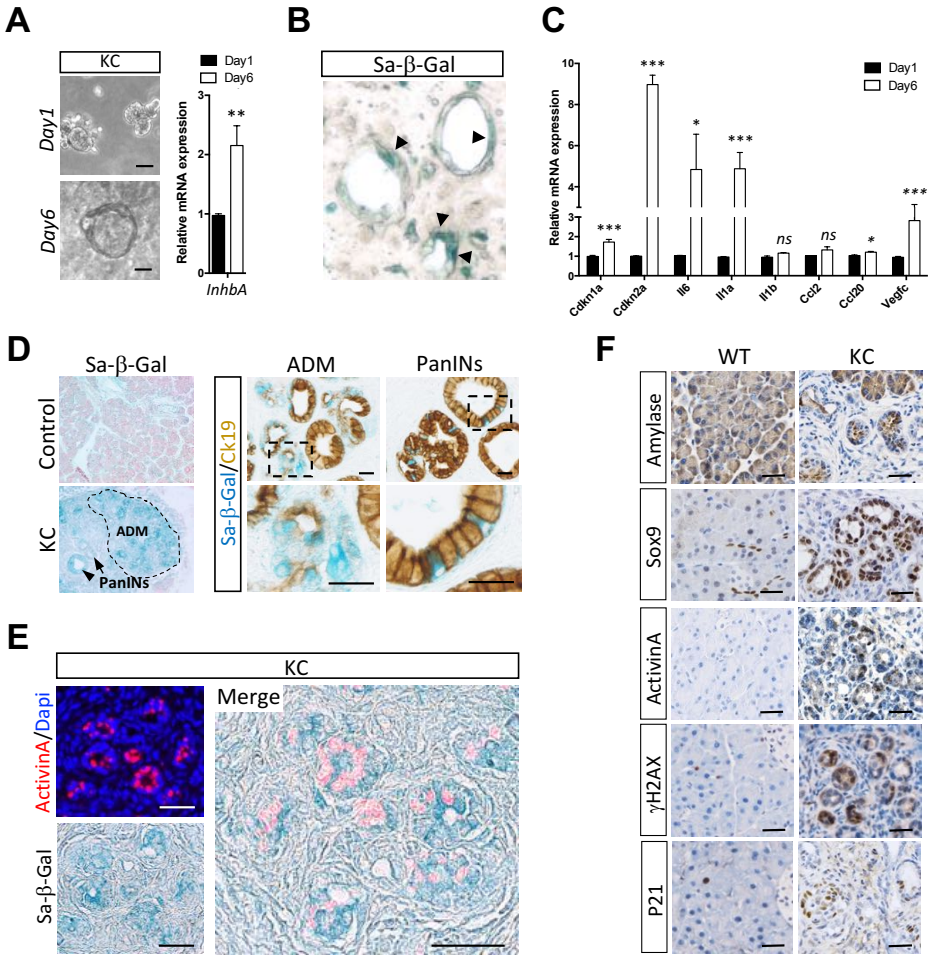


Figure 5, Zhao *et al.*

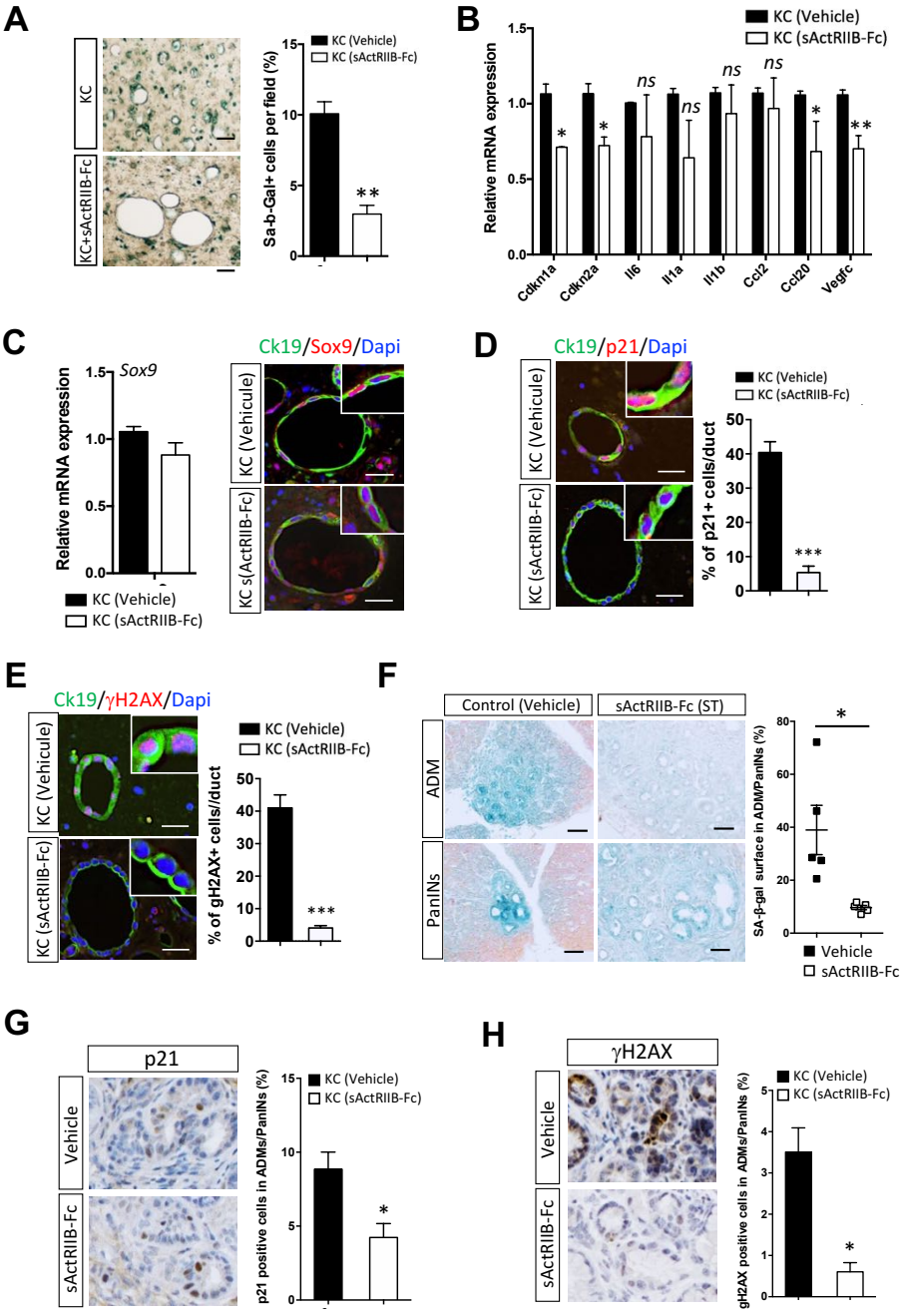


Figure 6, Zhao *et al.*

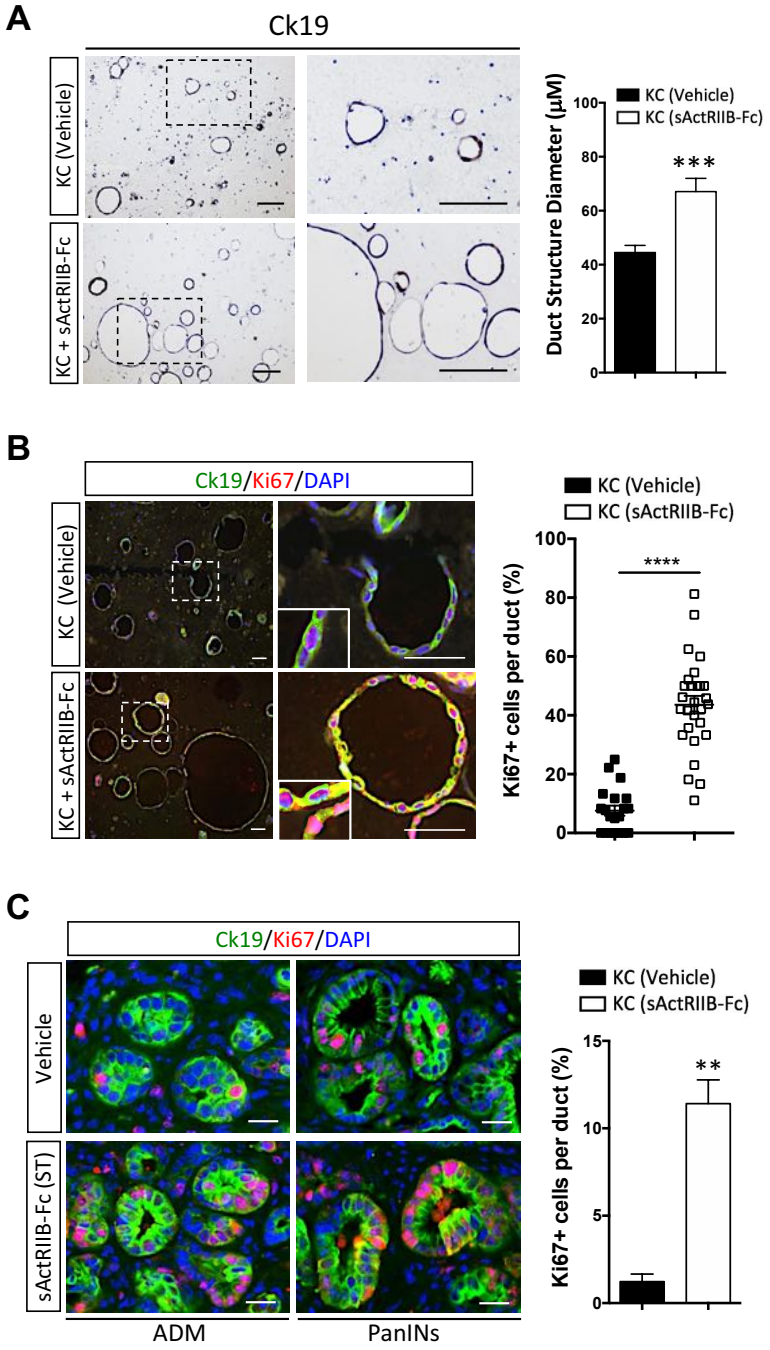
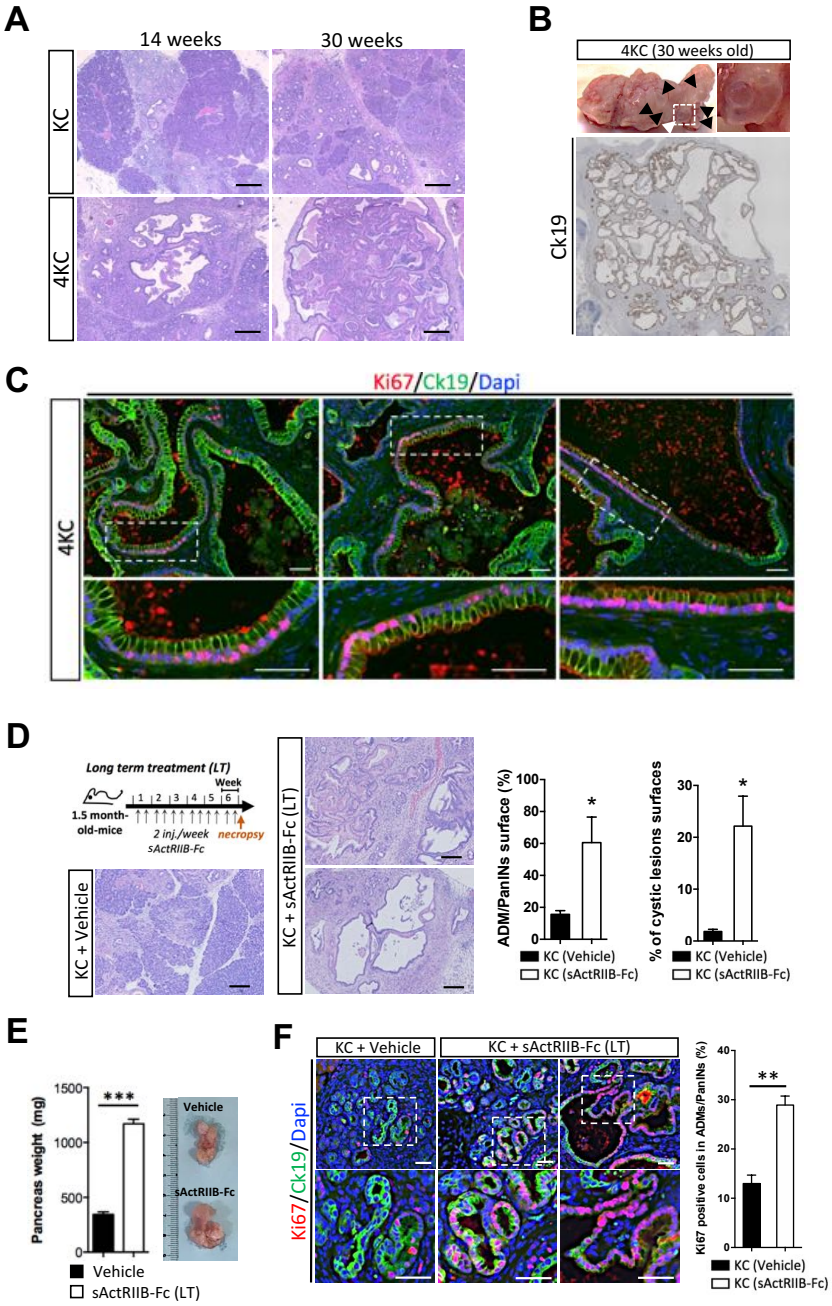
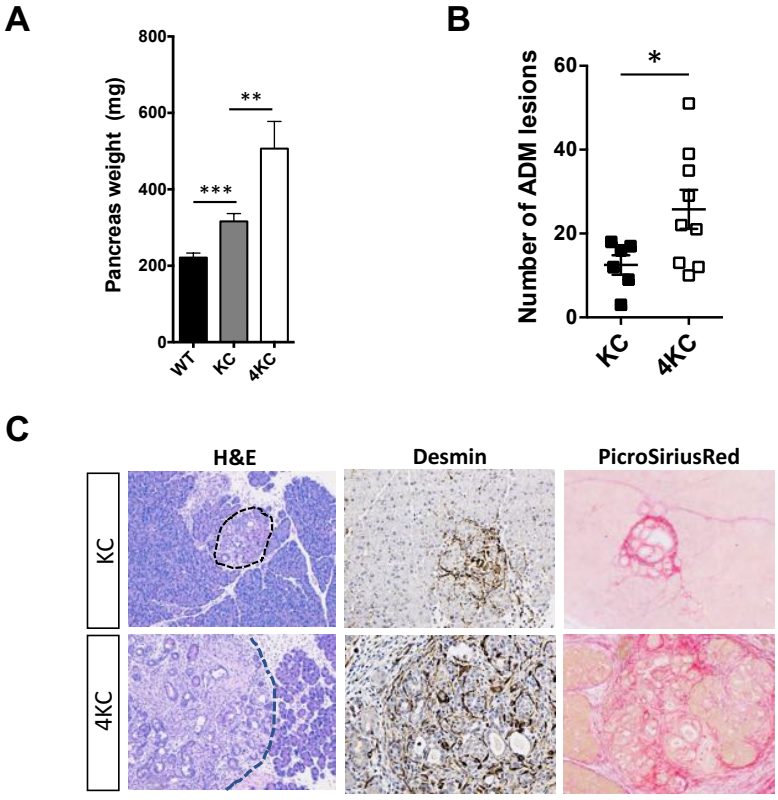


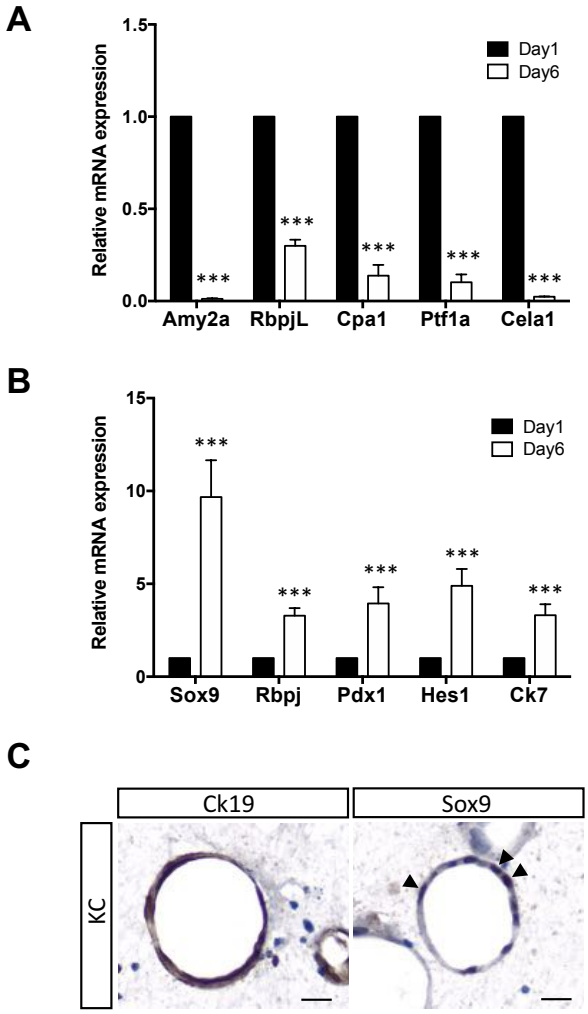
Figure 7, Zhao *et al.*

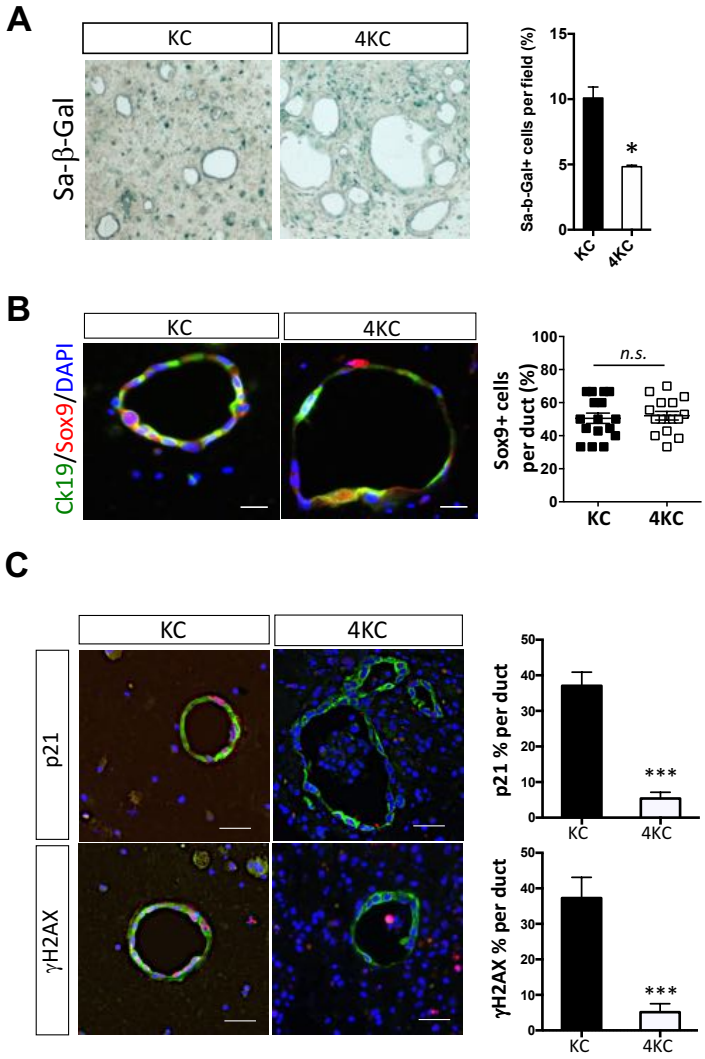


Supp. Fig. 1, Zhao *et al.*

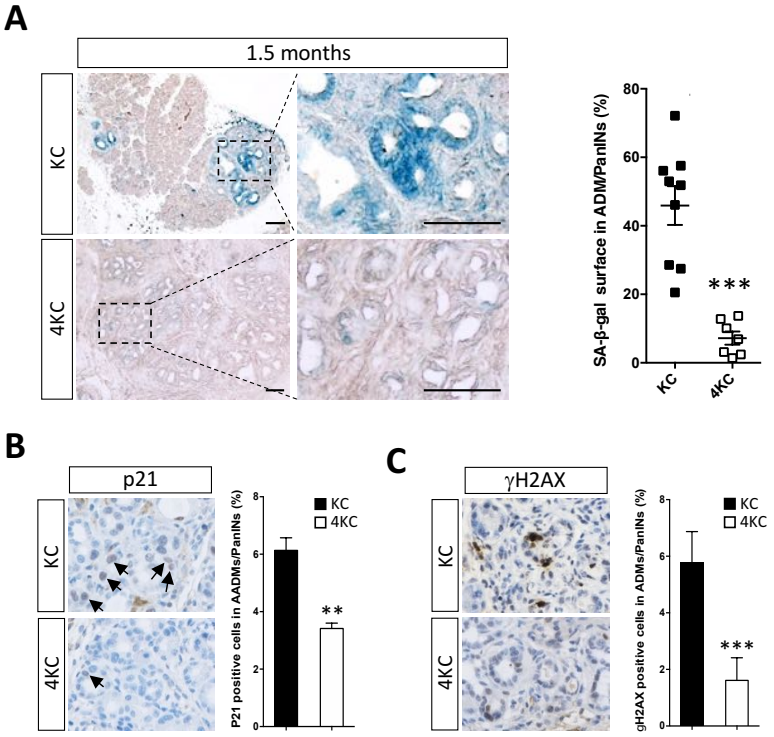


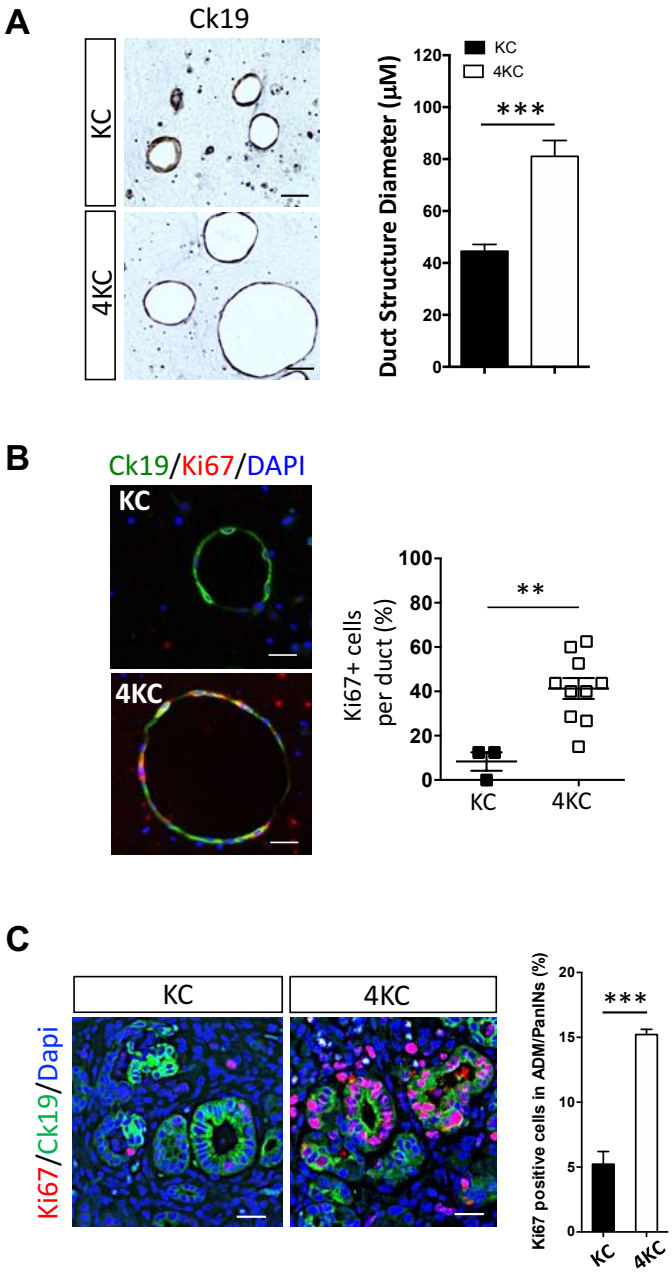
Supp. Fig. 2, Zhao *et al.*

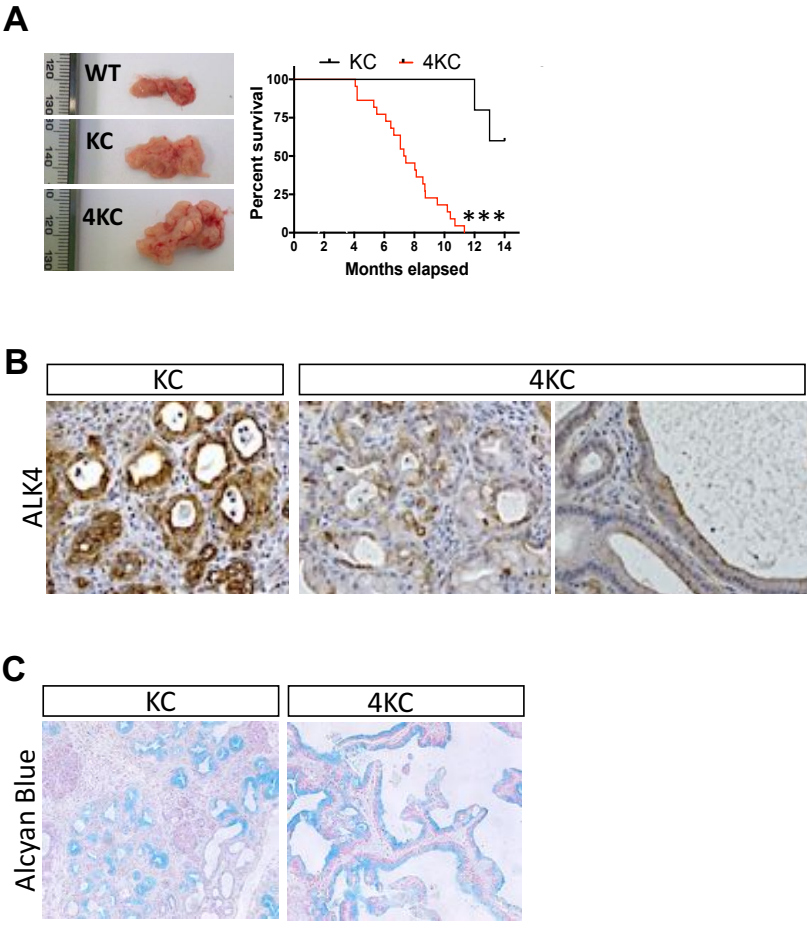



A
KC
4KC
Sa-β-Gal
Sa-b-Gal+ cells per field (%)
15
10
5
0
*
KC
4KC
B
KC
4KC
Ck19/Sox9/DAPI
Sox9+ cells per duct (%)
100
80
60
40
20
0
n.s.
KC
4KC
C
KC
4KC
p21
γH2AX
p21 % per duct
50
40
30
20
10
0
***
KC
4KC
γH2AX % per duct
50
40
30
20
10
0
***
KC
4KC

Supp. Fig. 4, Zhao *et al.*




A
Ck19
KC
4KC
KC
4KC
Duct Structure Diameter (µM)
120
100
80
60
40
20
0
***
B
Ck19/Ki67/DAPI
KC
4KC
Ki67+ cells per duct (%)
100
80
60
40
20
0
**
KC
4KC
C
KC
4KC
Ki67/Ck19/Dapi
Ki67 positive cells in ADM/PanINs (%)
20
15
10
5
0
***
KC
4KC


A
WT
KC
4KC
KC
4KC
Percent survival
100
75
50
25
0
0
2
4
6
8
10
12
14
Months elapsed
***
B
KC
4KC
ALK4
C
KC
4KC
Alcyan Blue

DISCUSSION

I Role of ActivinA and its signalling through ALK4 in the onset and progression of PDAC

1.1 ActivinA-signalling in controlling the progression and type of pancreatic precursor lesions

Indeed, although we talked about the role of ActivinA as a major ALK4-ligand in the preneoplastic lesions, the primary objective of my projet is based on the role of the lack of *ACVR1B* (ALK4) in the early stage of PDAC. *ACVR1B* is known to play an important role in development of PDAC. As early as in 1998, *ACVR1B* was found to be present in some subsets of PDAC by analyzing different pancreatic cancer lines (Kleeff *et al*., 1998). Later, based on the genetic evidence from human pancreatic cancer, *ACVR1B* was first referred as a tumor-suppressor gene (Su *et al*., 2001). In recent years, *ACVR1B* was suggested to be one of the candidate mutated genes that cooperate with $Kras^{G12D}$ to accelerate pancreatic tumorigenesis and promote progression in mouse and in human (Pérez-Mancera *et al*., 2012). Moreover, based on gene profiling, its role has also been emphasized in certain subtypes of PDAC (Bailey *et al*., 2016). On the other hand, Gloria's group revealed that the loss of *ACVR1B* in the context of oncogenic *Kras* mutation had a tendency in promoting IPMNs precursor development probably mediated by *NOTCH4* signalling pathway (Qiu *et al*., 2016). Similarly, as expected in our present study, our 4KC mutant animal using *Ptf1a-Cre* were found to develop CK19+ cystic lesions lacking ALK4 resembling the IPMNs reported within $Acvr1b^{flox/flox}$;$LSL\text{-}Kras^{G12D/+}$;*Pdx1-Cre* (AKP) model developed by Gloria's group. Furthermore, consistent with their study, we also observed an expansion of CK19+ cystic lesions in our 4KC GEMM is due to an increased proliferation in these lesions. Comparing with Gloria's study, although we share a very similar morphology and histology in the *ACVR1B* ablation mouse model, it still needs to pay close attention whether these CK19+ cystic lesions in our study are "real" IPMNs since the different *Cre* driver were used. Subsequent specific markers such as SOX17, MUC1, MUC2, MUC4, MUC5, MUC6 and CDX-2 may help to distinguish them (Basturk *et al*., 2010; Kitazono *et al*., 2013; Distler *et al*., 2014; Hong *et al*., 2012). Another

question worth further investigating is whether the lack of ActivinA-ALK4 signalling is a direct effect in remodelling these precursor lesions to become cystic, since we also found an accelerated formation of these similar CK19+ cystic lesions by using sActRIIB-Fc as an ActivinA ligand inhibitor. To that extent, using the ActRIIB-Fc in the 4KC mice will help to undertstand how much of the effect is due to blocking ALK4 signalling in acinar/duct cells with ALK4 ablation versus the systemic blocking of ALK4/ALK7 signalling in whole pancreatic tissue including the stroma as well as in the entire organism.

1.2 Role of other ALK4-candidate ligands during the onset of PDAC ?

Of note, while our work revealed the role of ActivinA as a key TGF β ligand in driving ALK4-signalling in low-grade lesions, we cannot firmly exclude that part of our observations could imply the contribution of other ALK4-ligands which signalling is blunted by the lack of ALK4 or through a sActRIIB-Fc mediated-inhibition. Among them, it is important to underline that the potent implications *of Inhbb* and *Gdf11* − 2 other ligands identified through our screen − should not be underestimated based on their sole weak induced-expression during these processes [Figure 1A]. Previous study from our group found *Inhbb* and its product ActivinB play a crucial role in controlling the plasticity of the endocrine-cell pancreatic tumorigenesis (Ripoche *et al*., 2015). In our study, we further observed an induction of ActivinB in the pancreatic islets of KIC and KC mice, which explains its moderate and stable expression over the time course of our screen analysis and suggests that it may impact the biology of pancreatic endocrine cells in the context of PDAC. To the same extent, *Gdf11* had previously been reported to restrain tumour growth by inducing apoptosis in pancreatic cancer (Liu *et al*., 2018). Yet those results were obtained from the use of tumour derived cell lines and the analysis of advanced tumour grades, it underlines the need to identify and characterize the cells that produce *Gdf11* in low-grade pancreatic lesions to better understand their role in the initiation of pancreatic cancer and the complexity of the intrinsic and extrinsic-signals that are mediated through ALK4. Besides, it is also worth paying attention to InhibinA and InhibinB, which are also ligands for ActRIIB and display a very slight expression at the beginning but not continuously express over the time course in our screen analysis, may have some implication on the early formation of low-grade lesions. Therefore, using more

specific inhibitor targeting ActivinA in KC mice may give us a more solid conclusion in our future work.

1.3 Contribution of ActivinA-ALK4 signalling in the late stage of PDAC

- Proliferation of tumor cells and stromal cells

Knowing that complex genetic changes occur in advanced stage of PDAC, I also addressed the question whether epithelial tumour cells derived from KIC and our 4KC GEMMs grow in the same pattern. Further analysis from my incucyte experiments showed that 4KC-GFP+ cells were grewing faster than KIC-GFP+ cells. By observing the similar gland-like structures formed in these xenograft tumors, I started to realize that ActivinA-signalling in advanced stage may not only drive different proliferative capacity of tumor cells, but could also impact the proliferation of stromal cells in a non-cell-autonomous manner. Based on our observation and the fact that tumor-produced ActivinA is secreted, the paracrine role of ActivinA-signalling is worth of being considered in the context of the tumor microenvironment. This point is further underlined by my results suggesting that grafted 4KC and KIC cells present different capacity to remodel their environement. Subsequent *in vitro* work suggests ActivinA-signalling may induce diverse molecules in KIC- and 4KC-GFP+ tumor cells and drive different paracrine signal in "educating" surrounding stromal cells. Another thing worth mentioning is that a crucial role of ActivinA-signalling is highlighted in the self-renewal of Pancreatic Cancer Stem Cells (PCSCs) and in creating a microenvironment favoring the tumorigenesis, which can be abrogated through the knockdown or pharmacologic inhibition of ALK4 and ALK7, implying ActivinA-signalling is a promising target for PCSCs therapy in the late stage of PDAC (Lonardo *et al*., 2011; Lonardo *et al*., 2012). Regarding these observations, I speculated whether different coexisted CSCs impact the proliferative capacity of both tumor cells and stromal cells under these two condition.

Indeed one may argue whether secretome produced by KIC tumor cells is dependent on ActivinA-signalling through ALK4. In this case, it would be of great interests to find out the candidate molecule produced in the PCSCs or non-PCSCs secretome that is regulated by downtream of ALK4-signalling and further comprehend its/their role in affecting cell growth, apoptosis, fibrosis and inflammation. To answer this question,

treating KIC PCSCs and non-PCSCs respectively with sActRIIB-Fc should help comprehend the cell autonomous role of ActivinA-signalling on tumour cells, while using the above cells condition media on the stromal cells, help undertanding the paracrine action of ActivinA. Depending on the observed results it might lead to the identification of ALK4-modulated secretome that could modulate the growth and functions of stromal cells. Results that could be subsequently studied through the used of our KIC- and 4KC-mouse and tumor-cell lines.

- Metastasis

TGF β -signalling has been reported to be pro-metastatic yet some publications have challenged this hypothesis and demonstrated it could show an anti-metastatic action (Levy and Hill, 2006; Lamouille *et al*., 2014). Although far less literatures focus on Activin-signalling and metastasis in PDAC, there are evidences showing Activin play a major role in such process (Cioffi *et al*., 2015; Togashi *et al*., 2015; Katoh and Katoh, 2010). From clinical studies, liver and lung metastasis as well as peritoneal carcinomatosis (including malignancy-related ascite) are manifestation of advance-progression of a variety of cancers. Once those lesions occur in PDAC, the overall median survival of patients do not exceed one year (Rochefort *et al*., 2019; Engin *et al*., 2013). During my PhD, I had been exploring the clinical association of our 4KC animals and compared it to KC and KIC mouse models, two other GEMMs commonly used in various lab to study the progression of PDAC. Interestingly, consistent with the observation from Gloria's group, our work found a diverse metastasis such as blood ascite and liver metastasis in 4KC GEMMs with an average survival time of 8~9 months (Qiu *et al*., 2016). Similarly to their results, we also detected a loss of p16 expression in 4KC animals with developed PDAC and their derived tumor cell lines through a PCR analysis of genomic DNA. This may suggest that the loss of p16 is indispensable for the progression of CK19+ cystic lesions in our 4KC mice to metastatic carcinomas (Qiu *et al*., 2016). On the other hand, KIC GEMMs has already been reported from previous study to be an aggressive PDAC model with a short survival time less than 8~12 weeks and an extensive invasion and metastasis in liver, spleen, duodenum, stomach and regional lymph nodes (Aguirre *et al*., 2003). Surprisingly, although we observed the same survival time in KIC GEMMs, it was not until recently that one of my collegue in our lab found lung metastasis in one KIC animal but not in the

cohort of my study. This made me questioned whether I could explore the differences between these two mouse models.

First, it is important to note that from a genetic point of view, ALK4 expression is ablated in 4KC-acinar cells while it is present in KC and KIC animals. Although consistent with Gloria's results that I also observed a loss of *p16* occurs in our 4KC mice, it is worth mentioning that *p16* inactivation occurs in KIC mice since embryo time while the loss of *p16* in 4KC mice is randomly acquired through the progression of the 4KC lesions. It is also important to mention that I found our KIC-derived tumor cell line to express ALK4 and ActivinA, while our 4KC-derived tumor cell line only expressed ActivinA. Knowing the importance of TGF β -signalling in the EMT (Levy and Hill, 2006), I next extented my analysis *in vitro* to determine whether the lack of Alk4 in the 4KC-cell line I used was impacting EMT mechanism. I gave a first try by using western blot to check some common EMT markers such as ZEB1, E-cadherin, Vimentin, Snail, Slug and Twist in KIC- and 4KC-derived tumor cell lines (Lamouille *et al*., 2014). Interestingly, while both of them behaved as epithelial cells marked with E-cadherin, I found a prominent expression of ZEB1 and a slight expression of Vimentin only in our 4KC-derived tumor cell line. However, I can not conclude as I only tested one 4KC-derived murine cell line. Besides, the downstream mechanism is far less studied at the moment. Testing the *in vivo* and *in vitro* (such as boyden chambers and migration assay) behaviour of the other three 4KC tumor cell lines generated in our lab will be important prior interpreting our datas. Characterization of human pancreatic cancer cell lines lacking ALK4 such as MIA-PaCa-2 and T3M4 may also be useful to comprehend the exact role of Alk4-signalling and and determine whether modulation of Alk4 may provide clinical benefits.

- Cachexia

Cancer-related cachexia represents a key determinant of cancer-related death. It has already been established that elevated levels of circulating ActivinA are asscociated with severe cachexia and poor outcome in various cancers and more recently senescence-associated aging conditions (Fearon *et al*., 2012; Han *et al*., 2013; Loumaye *et al*., 2015; Sartori *et al*., 2014; Alyodawi *et al*., 2019). In pancreatic cancer, it has also been reported that ActivinA-signalling is involved in cachexia through a mechanism involving ActRIIB-ALK receptor complex and the downstream SMAD effectors (Han *et al*., 2013;

Togashi *et al*., 2015; Parajuli *et al*., 2018). In my project, I noticed a high frequency of weight loss (about 50%) in both KIC and 4KC mice during long-term follow-up. Interestingly, the cause of weight loss is not necessarily the same. Based on the observation of the dissected pancreata, I considered the weight loss in our 4KC mice is mainly due to the compression of the extremely enlarged pancreas resulting in anepithymia, while in KIC mice it is more likely due to a real cachexia. Based on the severe loss of KIC mice, I did investigate whether blocking Alk4 signalling through the use of a sActRIIB-Fc injection impact tumor progression and overall weight loss. Based on the 3 animal included in treated vs non-treated group, the results were difficult to interpret as a biased outcome were seen in the treated group (one animal had a reduced tumour and a slightly extented survival but no increase in weight, while the phenotype were aggravated in another mouse with a slightly increased weight, and a third one died with a sudden weight loss). Regarding our preliminary results and the work from other people, the different results may firstly due to the mouse model we used. Parajuli *et al*. used KP53^{Luc} (*LSL-Kras*^{G12D}*; Trp53*$^{fl/+}$*;LSL-Luc; Pdx1-Cre*) and KP16^{Luc} (*LSL-Kras*^{G12D}*; p16Ink4a-Luc; Pdx1-Cre*) mouse model and had an average survival time of 30 to 36 weeks (Parajuli *et al*., 2018). The classic KIC (*Pdx1-Cre; LSL-Kras*^{G12D}*; Ink4a/Arf*$^{lox/lox}$) mouse model is a well-known aggressive PDAC model with an average survival time of 8 to 12 weeks and the inactivation of *p16* occurs during embryo time (Aguirre *et al*., 2003). Therefore, it is very possible that this after-birth inhibition of ActivinA-signalling is not sufficient enough to rescue the muscle weight loss and cachexia in such a short survival time period. In addition, it can not exclude that the rescue effect in both muscle cachexia and survival time seen in Parajuli's study may due to an incompleted mutation in *p16* and *p53*. In this context, a KPC mouse model (*PDX-1-Cre; LSL-Kras*^{G12D}*; LSL-Trp53*$^{R172H/-}$) with a longer survival time may be a better model to re-study whether the use of ActRIIB-Fc can also have a rescue effect in their survival time (Hingorani *et al*., 2005). Another thing needs to pay close attention is whether the use of such cachexia-therapy will affect PDAC growth. This question was also addressed in Parajuli's study, in which they demonstrated no effect was found on PDAC progression. However, in my project, although evidence is not solid and needs further investigation, I found an increased tendency in pancreas weight of KIC sActRIIB-Fc-treated group suggesting a possible acceleration of PDAC progression may occur.

II Role of Kras-OIS in the onset and progression of PDAC

2.1 SASP in PDAC: Is ActivinA a good SASP factor candidate in Kras-OIS during PDAC onset

TGF β superfamily ligands (*TGF β 1, INHBA, GDF15, BMP2 and BMP6*) have been reported to be candidate SASP components involved in paracrine senescence (Acosta *et al*., 2013). Subsequent *in vivo* and *in vitro* studies supported the idea that ActivinA may involved in TGF β -signalling mediated paracrine senescence. Xu *et al.* demonstrated that circulating levels of ActivinA could be a biomarker reflecting senescent cell burden *in vivo* (Xu *et al*., 2015). *In vitro* study from Haridoss *et al.* further indicated that growth inhibition of hepatocytes is mediated through autocrine and paracrine ActivinA regulated mechanisms resulting in a reduced proliferation capacity and an induction of senescence via $CDKN2B/p15^{INK4b}$ (Haridoss *et al*., 2017). Since *INHBA* gene (encoding for ActivinA) is a target of Kras oncogenic activation in pancreatic cancer, it is reasonable to speculate that ActivinA can be a SASP factor candidate in the initiation of PDAC (Qian *et al*., 2005). Interestingly, in our study, we observed that ActivinA was detected in SA-β-Gal+ cells within ADM and PanINs lesions with the expression of other two senescent markers (p21 and γ H2AX). Together with an induced expression of *INHBA* in acinar derived duct-structures subjected to senescence, our results support that ActivinA is a SASP factor that produced by senescent cells in ADM and low-grade PanINs.

Another thing needs to pay close attention is the underlying paracrine senescent effect of the other TGF β ligands mediated by Kras in the low-grade pancreatic neoplastic lesions and their surrounding microenvironment (Acosta *et al*., 2013). For example, in human breast cancer, the blockade of TGFβ signalling mediates the escape of oncogenic Ras-induced senescence and promotes tumorigenesis/metastatic transformation in mammary epithelial cells through the suppression of p21 (Lin *et al*., 2012). In a pancreatic mouse model, abrogation of TGFβ-signalling is also shown to suppress oncogenic Kras induced OIS in PanINs lesions (Acosta *et al*., 2013).

2.2 Possible role of ActivinA as a SASP

- Cytostatic modulator

While our data identify ActivinA and its signalling through ALK4 as a major contributor of the SASP program that limit ADM/PanIN progression, the cell autonomous and non-cell-autonomous molecular targets downstream of this signalling remain to be further defined. Previous studies have pointed the cytostatic mechanism mediated by ActivinA in other cancers. Panopoulou *et al.* used a neuroblastoma xenograft model and demonstrated that ActivinA acts as a cytostatic effect by inducing p21 through ALK4/SMAD pathway (Panopoulou *et al*., 2005). In breast cancer, ActivinA was also reported to inhibit cancer cell proliferation with a upregulation of p21 through a SMAD-signalling (Burdette *et al*., 2005). Furthermore, Grabliauskaite *et al.* found that loss of p21 increases ADM formation and senescence in the context of pancreatitis (Grabliauskaite *et al*., 2015). To the same extent, the inhibition of p21 suppresses Kras induced senescence and promote PDAC progression (Morton *et al*., 2010). In our study, we also found a paralelled reduced p21 and increased Ki67 expression in ADM/PanINs lesions under the inhibition of ActivinA-signalling. Therefore, together with these previous results, p21 can be a plausible target of ActivinA signalling in pancreatic epithelial cells in the onset of PDAC.

- Paracrine actions on stromal cells

Most of the previous studies showing TGF β /Activin-signalling plays a pro-fibrotic role in different cancers (Gonzalez-Gonzalez *et al*., 2017; Yin *et al*., 2017; Parker *et al*., 2017; Oh *et al*., 2012; Yamin *et al*., 2015; Protic *et al*., 2017; Kiagiadaki *et al*., 2018). In pancreas, ActivinA has been reported to have a dose dependent manner enhancing fibrosis in rat (Ohnishi *et al*., 2003). Surprisingly our results point that Dclk1 enriched lesions (hence possibly senescent cells producing ActivinA) yet surrounded by stromal region present a reduced amount of activated myofibroblasts marked by α SMA. It is known that cancer-associated fibroblasts (CAFs) are crucial components of stromal microenvironment in pancreatic cancer, originating from the activation of resident fibroblasts and PSCs, the differentiation of bone marrow-derived mesenchymal stem cells and EMT (Sun *et al*., 2018). The main source of CAFs in PDAC is PSCs (Vonlaufen *et al*., 2008). Activated CAFs exhibited myofibroblast-like characteristics positive for α SMA+ and other markers such as fibroblast-specific protein 1 (FSP1), platelet-derived growth factor receptor- α

(PDGFα) and β (PDGFβ), tenascin C, fibroblast activation protein (FAP), kindlin-2, paladin, fibronectin, integrin-α11, and podoplanin, with various expression levels in different CAF populations (Sun *et al*., 2018). TGFβ has been found to shape CAF heterogeneity in PDAC (Biffi *et al*., 2019). Based on these observations, although here I only mentioned the implication of ActivinA-signalling on αSMA+ activated myofibroblasts/CAFs, it is possible that this signalling can also impact other types of CAFs sited in the vicinity of ADM and PanINs lesions. On the other hand, one recent study revealed that stromal content is asscociated with tumor gland heterogeneity in human PDAC, which further impacts PDAC tumor biology, response to cytotoxic therapies and patient survival (Ligorio *et al*., 2019). In that case, it is still worth further investigating how ActivinA-signalling alters PDAC CAF heterogeneity and what its contribution in both tumor cells and stromal cells in PDAC onset.

- Paracrine actions on immune cells

With the arising of immunotherapy in PDAC patients, some interesting results pointed it as a promising field (Aroldi and Zaniboni, 2017). Although multiple evidences showed Activin-signalling is involved in the control of immune functions in many cancers, its role in regulating the immune surveillance and compartment is not studied in PDAC (Ogawa and Funaba, 2011; Hedger *et al*., 2011). In this case, it is worth paying attention to the portent role of ActivinA-signalling on the regulation of immune functions. In addition, based on the previous report that Activin-signalling can regulate the polarization of macrophage which further promotes PDAC progression (Sainz *et al*., 2015), it suggests that ActivinA paracrine action might be more complicated than initailly thought. Since many SASP factors have been known as the pro-inflammatory actor, it is of great interests to further investigate whether ActivinA is a pro- or anti-inflammatory SASP factor in PDAC microenvironment besides its cytostatic effect in tumor cells (Perrott *et al*., 2017; Kuilman *et al*., 2008; Hoare *et al*., 2016).

2.3 Senescent cells in ADM and PanINs

My work report that SA-β-Gal activity and ActivinA expression were increased in a large population of cells within ADM, while only a limited number of cells present those characteristic in PanINs. This observation is puzzling and may support as a possible explanation, the existence of subtypes of senescent cells. Indeed one may argue that senescent cells in ADM and in PanINs may differ regarding their origin and their respective functions. In this context, it made me think whether ActivinA produced by senescent cells in ADM and PanINs serves the same role, whether senescent cells in ADM and PanINs have different identities secreting two distinct secretomes and what the underlying mechanisms are that reduce and restrict senescence to a subset of cells among these low grade lesions.

Indeed, there is always this kind of debate about the role of SASP either serves as an anti-tumorigenic or a pro-tumorigenic role in the preneoplastic cells and their surrounding microenvironment. In recent years, the dynamic pattern of Ras-related SASP has been proposed to be involved in this process driven by distinct signalling cascades at various points in time after senescence induction, accounts for early pro-senescent immunosuppression that can evolve into immunogenic, pro-inflammatory and profibrotic effects (Xue *et al*., 2007; Hoare *et al*., 2016; Acosta *et al*., 2013). Based on this new insight of SASP during tumor formation, it helps to explain our results showing that Dclk1 positive senescent cells in ADM and PanINs lesions produce ActivinA as a pro-senescent SASP in limiting the expansion of low grade lesions and reducing the activation of α-SMA positive cells in the microenvironment, could due to the engagement of ActivinA in the "first wave" secretome through a different downstream signalling (probably NOTCH1/TGF β 1), while in a later time, ActivinA and other SASPs such as IL13 and Cxcl2 reported by previous studies more likely involved in the "second wave" secretome act as pro-inflammatory or profibrotic effect (Lee and Schmitt, 2019; Liou *et al*., 2017; Shao *et al*., 2019).

Furthermore, another question I want to address is whether ActivinA still acts as a SASP factor in advanced stage of PDAC. Indeed, I do not have solid evidence at the moment showing senescent cells still exist in advanced lesions, although I did observed a number of senescent cells present in low grade PanINs. In this case, performing a whole-mount SA-β-Gal staining on the KIC and the aging 4KC GEMMs could help to solve this doubt and eventually identify a subset of senescent cells in the close vicinity of

PDAC that may contribute through their SASP to tumor behaviour and outcome. Even senescent cells might be present in advanced lesions, I am not sure whether these cells will still produce ActivinA. While the mechanisms driven the diffuse production of ActivinA in advanced stage need to be further comprehend, it opens the questions of ActivinA driven actions and whether those are cell autonomous and/or modulated through paracrine effects.

2.4. SAS in PDAC

Another important thing worth paying close attention is the impact of senescence on stemness in PDAC. While some previous studies showed that induced senescence in mouse mammary epithelial stem cells or in human mesenchymal stem cells decrease the risk of developing cancer (Boulanger and Smith, 2001; Serakinci *et al*., 2004), some others debated that senescence in stem cells promote tumorigenesis through the exhaustion of stem cells pools and the disruption of stem cell microenvironment (Rudolph *et al*., 1999; Wong and Collins, 2003). Since some SASP factors have been reported to be associated with CSCs, it is reasonable to think that these secretome act as a bridge between CSCs and tumor microenvironment (Iliopoulos *et al*., 2011; Kim *et al*., 2013; Charafe-Jauffret *et al*., 2009). Furthermore, most of the litteratures support that senescence promotes cancer stemness through the proinflammatory or profibrotic SASP factors. For example, a recent study reported that a number of proinflammatory SASP factors including macrophage migration inhibitory factor (MIF), HGF, and chemokine C-C motif ligand 2 (CCL2) help to enhance cancer stemness in colon cancer (Muñoz-Galván *et al*., 2019). Liou *et al*. also demonstrated that IL13 a SASP factor is produced by a subset of Dclk1+ cells within low grade pancreatic neoplastic lesions and acts as a profibrotic role through a macrophage-mediated inflammatory responses (Liou *et al*., 2017). In our work, we also detected these Dclk1 positive cells in low grade lesions. Interestingly, although Dclk1 has been recognized as a quiescent or tumor initiating stem cell marker in previous studies (Nakanishi *et al*., 2013; May *et al*., 2008), our work suggests that Dclk1 positive stem cells in ADM and PanINs can also be senescent under some condition and the senescent phenotype of Dclk1 postive cells can be sustained by ActivinA-signalling. Further observation showing Dclk1 enriched lesions display a reduced proliferation in tumor cells and a reduced α SMA+ CAFs regulated by ActivinA-signalling both in an autocrine and in a paracrine way. Combining the results from Liou *et al*. and ours, it might support the fact that senescent cells in ADM and in PanINs are very different. Considering the dynamic

pattern of Ras-related SASP, it is very possible that SASP factors act as a prosenescent or protective role in ADM, while a proinflammatory or profibrotic role in PanINs.

More interestingly, our study found that Ki67-proliferative cells lining in strips within the precursor lesions frequently sited next to the Dclk1 positive cells in mice lack of ActivinA-signalling. One may question if the lack of ActivinA-signalling might change the behaviour/fate of Dclk1+ senescent cells and whether to those later cells have a paracrine action (i.e tumor-promoting) on tumour epithelial cells, which could explain the highly proliferated CK19+ cystic lesions formed in our 4KC and sActRIIB-Fc-treated KC mice.

III Clinical perspective of the work

3.1 ActivinA as a novel diagnostic tool for low-graded pancreatic tumors ?

As ActivinA is a secreted protein and had been shown to circulate in the blood, it is reasonable to think that ActivinA could be used as a diagnostic tool from a clinical point of view. In fact, this idea is not totally new since previous studies have already reported that ActivinA is expressed by a subset of human pancreatic cancer and a series of derived cell lines (Kleeff *et al*., 1998; Togashi *et al*., 2015). Moreover, it was proposed that plasma ActivinA level can be a novel prognostic factor in patients with advanced PDAC by setting its cut-off at 800pg/ml determined using an ELISA assay (Togashi *et al*., 2015). Based on these results, they considered that ActivinA has a continuously increased expression during the progression of PDAC. Our work support that ActivinA plasma dosage might also serve the identification of preneoplastic pancreatic lesions base on its high expression in low-grade ADM and PanINs. However it will be important to determine whether it would be possible to detect the ActivinA produced by a small number of ADM lesions compared to the quantity a large tumor may produce. To that extent, I do believe that ActivinA detected would certainly serve the identification of pancreatic cancerous lesions, but would not necessarily allow a precise grading of the tumor stage the patients are in. Although the expression level of ActivinA in lesions may not exactly be consistent with its plasma level, it still worth trying to define a reference range and test the sensitivity/specificity of ActivinA according to different stage of PDAC. Such work could be envisioned through the use of our KC and KIC GEMMs and commercially available detection kit for ActivinA.

3.2 Targeting Activin-Signalling for therapeutic applications: a good or a bad idea?

By comparing my work to the previous reported role of ActivinA, it is now evident that the role of ActivinA in PDAC is complex, impacting tumor cells as well as their microenvironement and appears to be protection but also favoring the onset and progression of PDAC. My PhD work underline ActivinA plays an anti-tumoral role in the initiation of PDAC through its function as a Kras-promoted SASP. To that extent, it could be appealing to consider applying ActivinA treatment strategy to prevent the progression of pre-neoplastic lesions. However, this is not a feasible strategy to conduct at the moment as effective methods for early detection in PDAC patients are still lacking. In this case, the timing for the use of ActivinA applications/injections may appear uncertain. More importantly injecting ActivinA could be deleterious, knowing ActivinA has been reported to participate in rat pancreatic fibrosis (Ohnishi *et al*., 2003) and is considered as a pro-fibrotic cytokine in other organs such as uterus and liver (Protic *et al*., 2017; Kiagiadaki *et al*., 2018). To that extent, systemic use of ActivinA may not be recommended for limiting PDAC onset but on the potent numerous side-effects.

More importantly, my PhD work suggest that targeting Activin-signalling through the use of a systemic approach involving soluble form of ActRIIB-Fc receptor is not devoid of risk to favor the progression of preneoplastic pancreas lesions into PDAC. This comment is very important to my eyes as targeting ActivinA through the use of anti-ActRIIB blockade strategies has been proposed as an effective intervention against muscle atrophic-conditions and cancer cachexia and more recently senescence-associated aging conditions (Alyodawi *et al*., 2019). Promising therapeutic benefits have been shown on reverting cancer-cachexia promoted by ActivinA or Myostatin production by xenografted lung and colon cancer cell lines (Busquets *et al*., 2012; Nissinen *et al*., 2018). Our results pinpoint that the use of such cachexia-therapy, while efficient in many cancers and pathological-conditions, should be considered with extreme caution in the case of pancreatic cancer. Indeed, knowing that low grade pancreatic lesions are frequently found in peri-tumoral regions surrounding PDAC, one may question whether the benefit of blocking ActivinA-induced cachexia through a systemic approach based on the use of ActRIIB-Fc strategy won't favour the progression of neoplastic lesions into more advanced grades. While such treatment may represent a promising strategy for patients with

pancreatic tumour mutated for ALK4 or SMAD4, next-gen tissue-specific therapeutic delivery should be considered for others to avoid the potent deleterious effect of using ActRIIB-Fc.

CONCLUSION AND PERSPECTIVE

To conclude, in the first part of my project, we uncover a novel role of ActivinA in the initiation of PDAC. Our work provides the first demonstration that ActivinA and its signalling through ALK4 represent one of the first defence that is induced through Kras-OIS to limit the development and progression of ADM/PanINs.

By further exploring the autocrine and paracrine role of ActivinA-signalling in our i*n vitro* and *in vivo* experimental models, my work also pointed that lack of ActivinA-ALK4 signalling in mice leads to a decreased number of Dclk1+ cell number among lesions and a different proliferative capacity of both tumor cells and their surrounding stromal cells in more advanced tumour grades.

For the perspective of my work, several questions remain to be addressed, such as:

i) whether the lack of ActivinA-ALK4 signalling is a direct effect in remodelling these precursor lesions to become cystic;

ii) whether the functions of other ALK4-ligands contribute to the initiation of PDAC;

iii) to find out the candidate molecule produced in the PCSCs or non-PCSCs secretome that is regulated by downtream of ALK4-signalling and further comprehend its/their role in affecting cell growth, apoptosis, fibrosis, inflammation and metastasis;

iv) to exclude the underlying paracrine senescent effect of the other TGF β ligands mediated by Kras in the low-grade pancreatic neoplastic lesions and their surrounding microenvironment;

v) to define the cell autonomous and non-cell-autonomous molecular targets downstream of this signalling;

vi) to investigate how ActivinA-signalling alters PDAC CAF heterogeneity and what its contribution in both tumor cells and stromal cells in PDAC onset.

From a clinical perspective, my work also bring a number of important questions that should be explored. Use of ActvinA as a diagnostic tool might be envisioned following a proper evaluation of the reference range and the sensitivity/specificity of serum ActivinA according to different stage of PDAC. More importantly, my work highlight that therapeutic application through a systemic use of ActivinA should not be recommended for limiting PDAC onset due to the potential side-effects. Targeting ActivinA through the use of anti-ActRIIB blockade strategies should therefore be considered with extreme caution in the case of pancreatic cancer, but may represent a promising strategy for patients with pancreatic tumour mutated for ALK4 or SMAD4.

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APPENDICES (List of poster teaser and poster)

Zhao Y., Goehrig D., Nigri J.,Zhang X., Vasseur S., Tomasisni R., Hennino A. and Bertolino P. Loss of Alk4 impact the onset of pancreatic cancers through an active pancreas remodeling and an induced proliferation of Acinar to Ductal metaplastic (ADM) lesions. 2017, 09. The 3rd International Cancer Symposium of the Cancer research center of Lyon. **(Poster teaser)**

Zhao Y., Goehrig D., Nigri J.,Zhang X., Vasseur S., Tomasisni R., Hennino A. and Bertolino P. Loss of Alk4 impact the onset of pancreatic cancers through an active pancreas remodeling and an induced proliferation of Acinar to Ductal metaplastic (ADM) lesions. 2017,12. Journee Scientifique de La SFR Sante Lyon Est. **(Poster)**

Zhao Y., Goehrig D., Nigri J.,Zhang X., Vasseur S., Tomasisni R., Hennino A. and Bertolino P. Loss of Alk4 impact the onset of pancreatic cancers through an active pancreas remodelling and an induced proliferation of Acinar to Ductal metaplastic (ADM) lesions. 2018, 03. International ADELIH Congress 2018 Paris. **(Poster)**