大学院 自然科学研究科 | Graduate School of Natural Science

Studies on iPSCs-converted pancreatic Cancer Stem Cells

博士課程 | Doctoral course

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Index

To all the warriors and their beloved relatives that have had faced this disease.

All my support and affection.

A tots els lluitadors i lluitadores i als seus estimats familiars que han hagut d'enfrontar aquesta malaltia.

Tot el meu suport i afecte.

A todos los luchadores y luchadoras, y a sus queridos familiares que han tenido que afrontar esta enfermedad.

Todo mi apoyo y afecto.

Summary

Pancreatic ductal adenocarcinoma (PDAC) represents the 90% of the forms of pancreatic cancers. The mortality rate is high due to its rapid dissemination, the strong resistance to the radio and chemotherapy, along with the lack of prognostic approaches. Unfortunately, a merely 20% out of the total diagnosed patients may go under treatment and the 5-year survival rate is less than 5%. Therefore, this highlights the urgent need to find novel therapies along with new pancreatic cancer markers towards the early detection of the primary stages of the PDAC.

PDAC solid tumours are constituted by heterogeneous populations. Cancer stem cells (CSC) are a unique subpopulation of cells within the tumour that possess stem properties such as self-renew, but also are able to give rise a diverse progeny with self-limited proliferative capacity which perpetuates the malignant growth and even promotes the invasion towards new tissues. These particular characteristics place them at the apex of an organized hierarchical system where CSC are responsible for the new generation of more differentiated progenies (non-CSC) and are accompanied by desmoplastic reactive stroma and immune cells.

The identification and consequent isolation of pancreatic CSCs facilitated the generation of genetically engineered murine models. Nonetheless, the current models may not be representative for the spontaneous tumour occurrence.

The present study show the generation of a novel pancreatic iPSC-converted cancer stem cell lines (CSCcm) as a cutting-edge model for the study of PDAC. Thus, CSCcm lines were exclusively achieved by means of the conversion of iPSCs using the influence of pancreatic cancer cell line PK-8 and KLM-1 conditioned medium (CM) and were not subjected to any genetic manipulation.

The xenografts tumours resulting from both the subcutaneous and the orthotopic transplantation of CSCcm PK8 and CSCcm KLM-1 cell lines displayed histopathological adenocarcinoma-like morphologies and showed pancreatic cancer specific hallmarks as PanIN lesions and PDAC features. In addition, when CSCcm cell lines were orthotopically implanted

not only recapitulated the histological features of pancreatic cancer in the primary tumours but also displayed a preferential metastasis towards the liver as the current disease. Consistently, immunohistochemistry analysis for the lineage tracing marker GFP demonstrated that either the pancreatic lesions or the metastatic nodes were originated from CSCcm cell lines.

A preliminary screening for the expression of the transcript levels for the most representative CSC markers revealed a strong enhancement for the primary culture CSCcm cell lines with a slight decrease in the subsequent generations, suggesting a possible establishment of a more differentiated phenotype. This was further confirmed by the molecular characterization from RNA-sequencing analysis which indeed highlighted primary culture cell lines (1^{st} CSCcm) as potential candidates to represent the pancreatic CSCs and indicated the establishment of a more differentiated pancreatic cancer molecular pattern in their subsequent progenies 2^{nd} CSCcm and 3^{rd} CSCcm.

It is noteworthy that RNA-seq analysis revealed that the distinct CSCcm lines did not harbour single point mutations for the oncogene Kras codon 12 or 13 and that DESeq analysis showed increased expression of Myc in the 2^{nd} CSCcm generation whilst it was remarkably downregulated in 1^{st} CSCcm. The latter correlated with the recent findings on genome-wide DNA methylation profiling which determined CpG sites annotated to Myc to be highly methylated in pancreatic CSCs rather than in more differentiated pancreatic cancer cells. Furthermore, the overexpression of the Dnmt3a and Dntm3b observed in 1^{st} CSCcm and the subsequent downregulation in 2^{nd} CSCcm strongly suggested that the activation of Kras and Myc are required but not sufficient to originate a PDAC tumour and their turnover between 1^{st} CSCcm and more differentiated 2^{nd} CSCcm may be tightly related to epigenetic alterations.

Even though the conditioned medium from cancer cell lines provides an appropriate microenvironment able to initiate a malignant transformation it is not until CSCcm get in touch with an *in vivo* system that the CSCs features become robust at the level of transcriptome. Therefore, the intervention of the organisms is still essential. Certainly, this reliance on *in vivo* systems is an unresolved matter that must eventually be overcome.

Overall, we conclude that PDAC-CSCcm model may provide new insights about the actual occurrence of the pancreatic cancer leading to develop different approaches to target CSCs and abrogate the progression of this fatidic disease.

Chapter 1

Introduction to Pancreatic Cancer

Pancreatic cancer

Epidemiology

Pancreatic ductal adenocarcinoma (PDAC) the predominant form of pancreatic cancer is the 6th leading cause of cancer-related death (6% of all cancer deaths) in Europe, with 104,481 estimated deaths in 2012. The 5-year survival rate for PDAC is less than 5% (Figure 1).

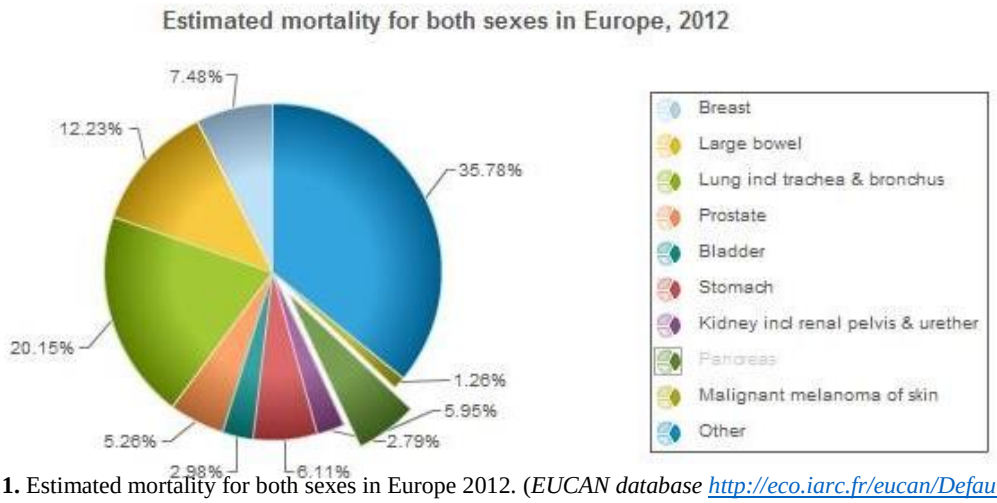

Figure 1. Estimated mortality for both sexes in Europe 2012. (*EUCAN database* http://eco.iarc.fr/eucan/Default.aspx *2012 Eupancreas*).

According to mortality predictions for the year 2015 in the European Union, mortality of pancreatic cancer will keep increasing between 2009 and 2015 in both sexes (rising by 4% in men and 5% in women), becoming the fourth leading cause of cancer death with 8% and 6% of all cancer deaths in men and women, respectively (1). Projections of cancer incidence and mortality to 2030 also reveal that pancreatic cancer will become the second leading cause of cancer-related death in the US (2).

Risk factors for PDAC

The potential risk factors for developing pancreatic cancer are the age, gender, race, smoking, diabetes, familial history, inflammation of the pancreas, obesity and other risk factors mainly related to diet and genetic syndromes that are under active study (3, 4).

- Age: The risk of developing pancreatic cancer goes up as people age. Almost all patients are older than 45. About two-thirds are at least 65 years old. The average age at the time of diagnosis is 71.
- Gender: Men are slightly more likely to develop pancreatic cancer than women. This may be due, at least in part, to higher tobacco use in men, which raises pancreatic cancer risk (see above). The difference in pancreatic cancer risk was larger in the past (when tobacco use was much more common among men than women), but the gap has closed in recent years.
- Race: African Americans are slightly more likely to develop pancreatic cancer than whites. The reasons for this aren't clear, but it may be due in part to having higher rates of some other risk factors for pancreatic cancer, such as diabetes, smoking in men, and being overweight in women.
- Smoking: Pancreatic cancer risk is 2.2 times higher in current smokers compared with never-smokers, a pooled analysis showed. Risk increases with the number of cigarettes smoked per day, and duration of cigarette smoking, meta- and pooled analyses have shown.
- Diabetes: Pancreatic cancer is more common in people with diabetes. The reason for this is not known. Most of the risk is found in people with type 2 diabetes. This type of diabetes most often starts in adulthood and is often related to being overweight or obese. It's not clear if people with type 1 (juvenile) diabetes have a higher risk.
- Family history: Pancreatic cancer seems to run in some families. In some of these families, the high risk is due to an inherited syndrome (explained below). In other families, the gene causing the increased risk is not known. Although family history is a risk factor, most people who get pancreatic cancer do not have a family history of it.
- Inflammation of the pancreas: Chronic pancreatitis, a long-term inflammation of the pancreas, is linked with an increased risk of pancreatic cancer (especially in smokers), but most people with pancreatitis never develop pancreatic cancer. Chronic pancreatitis

is sometimes due to an inherited gene mutation. People with this inherited (familial) form of pancreatitis have a high lifetime risk of pancreatic cancer.

- Obesity: Body fatness is classified by the International Agency for Research on Cancer (IARC) and the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) as a cause of pancreatic cancer. Abdominal fatness is classified by WCRF/AICR as a probable cause of pancreatic cancer. Pancreatic cancer risk increases by 10% per 5-unit body mass index (BMI) increase, a meta-analysis showed. Pancreatic cancer risk increases by 11% per 10cm waist circumference increase, and by 19% per 0.1-unit waist-to-hip ratio increment, this meta-analysis showed.
- Genetic syndromes: Inherited gene changes (mutations) can be passed from parent to child. These gene changes may cause as many as 10% of pancreatic cancers. Sometimes these changes result in syndromes that include increased risks of other cancers (or other health problems).

Histology of Pancreatic cancer

The pancreas is an organ located behind the stomach in the upper left abdomen. It is surrounded by other organs including the small intestine, liver, and spleen. It is spongy, about six to ten inches long, and is shaped like a flat pear or a fish extended horizontally across the abdomen. The wide part, called the head of the pancreas, is positioned toward the center of the abdomen at the juncture where the stomach meets the first part of the small intestine (Figure 2).

It has two main functions: the exocrine which involves the acinar cells secreting enzymes that are transported into the small intestine by the pancreatic duct and are important to digestion and the endocrine that consists of islet cells (islets of Langerhans) that create and secrete important hormones such as glucagon, insulin, somatostatin, and pancreatic polypeptide (PP) directly into the bloodstream.

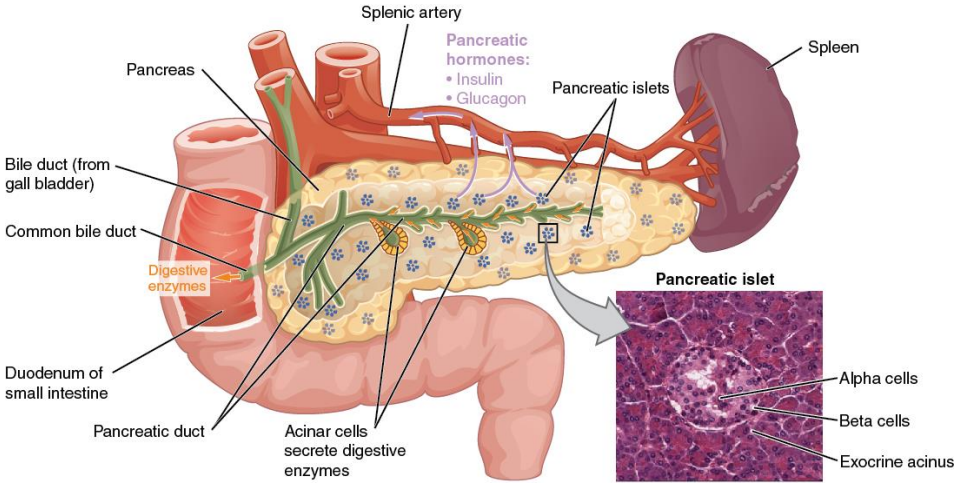

Figure 2. The pancreatic exocrine function involves the acinar cells secreting digestive enzymes that are transported into the small intestine by the pancreatic duct. Its endocrine function involves the secretion of insulin (produced by beta cells) and glucagon (produced by alpha cells) within the pancreatic islets. These two hormones regulate the rate of glucose metabolism in the body. The micrograph reveals pancreatic islets. LM × 760. (Micrograph provided by the Regents of University of Michigan Medical School © 2012). (*The Endocrine System. Anatomy and Physiology. OpenStax*).

The majority of exocrine tumours found in the distal part of the pancreatic gland are adenocarcinomas. Commonly are differentiated as pancreaticobiliary adenocarcinomas resembling pancreaticobiliary duct epithelium, although less frequently found, they also may differentiate as intestinal type displaying intestinal/colon-like phenotype. Adenocarcinoma in the pancreas has a tendency to produce fibrous connective tissue making them very hard tumours.

It has long been considered that pancreatic ductal adenocarcinoma arise from pancreatic ducts on the basis of its morphology, the occurrence of dysplasia in putative preneoplastic ductal lesions, and the absence of acinar dysplasia in the pancreas of patients with PDAC. However, evidence gathered through both in vitro studies and more importantly genetic mouse models of PDAC shows that ductal-type tumours can arise from acinar cells. These findings raise new important questions related to PDAC pathophysiology and call for in-depth studies of acinar cell differentiation in order to better understand PDAC biology (5).

Three histologically defined preneoplastic precursor lesions to invasive adenocarcinoma of the pancreas have been identified. These include microscopic, non-cystic pancreatic

intraepithelial neoplasia (PanIN) and macroscopic, cystic intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN) (6, 7).

Based on the international nomenclature and classification established by the assembly of Pancreatic Cancer Think Tank held in Park City, Utah in September 1999, we can currently define the preneoplastic lesions as following (7-10):

- PanIN-1A: (Pancreatic Intraepithelial Neoplasia 1-A): These are flat epithelial lesions composed of tall columnar cells with basally located nuclei and abundant supranuclear mucin. The nuclei are small and round to oval in shape. When oval the nuclei are oriented perpendicular to the basement membrane. It is recognized that there is considerable histologic overlap between non-neoplastic flat hyperplastic lesions and flat neoplastic lesions without atypia. Therefore, some may choose to designate these lesions with the modifier lesion ("PanIN/[L]-1A") to reflect the fact that the neoplastic nature of many cases of PanIn-1A has not been established.
- PanIN-1B: (Pancreatic Intraepithelial Neoplasia 1-B): These epithelial lesions have a papillary, micropapillary or basally pseudostratified architecture, but are otherwise identical to PanIN-1A.
- PanIN-2: (Pancreatic Intraepithelial Neoplasia 2): Architecturally these mucinous epithelial lesions may be flat or papillary. Cytologically, by definition, these lesions must have some nuclear abnormalities. These abnormalities may include some loss of polarity, nuclear crowding, enlarged nuclei, pseudo-stratification and hyperchromatism. These nuclear abnormalities fall short of those seen in PanIN-3. Mitoses are rare, but when present are non-luminal (not apical) and not atypical. True cribriforming luminal necrosis and marked cytologic abnormalities are generally not seen, and when present should suggest the diagnosis of PanIN-3.
- PanIN-3: (Pancreatic Intraepithelial Neoplasia 3): Architecturally, these lesions are usually papillary or micropapillary, however, they may rarely be flat. True cribriforming, budding off of small clusters of epithelial cells into the lumen and luminal necroses

should all suggest the diagnosis of PanIN-3. Cytologically, these lesions are characterized by a loss of nuclear polarity, dystrophic goblet cells (goblet cells with nuclei oriented towards the lumen and mucinous cytoplasm oriented toward the basement membrane), mitoses which may occasionally be abnormal, nuclear irregularities and prominent (macro) nucleoli.

- Intraductal Papillary Mucinous Neoplasms (IPMNs): IPMNs are mucinous epithelial neoplasms which involve the main pancreatic duct or its major branches. They are larger than PanINs and therefore usually visible grossly or by radiologic imaging. IPMNs may extend into small ducts. In these cases serial (step) sections may be helpful in defining the relationship of the two lesions.
- Mucinous cystic neoplasms (MCN): Mucinous cystic neoplasms are characterized by the presence of ovarian stroma and the absence of a connection to the duct system. These features and the larger size of mucinous cystic neoplasms help distinguish mucinous cystic neoplasms from PanINs.

Key regulators in PDAC carcinogenesis

The accumulation of somatic mutations, epigenetic modifications and changes in the micro-environment leads to the development of pancreatic cancer.

Somatic mutations together with genomic rearrangements have been identified to drive tumorigenesis in pancreatic cancer (11, 12). The mutation in KRAS codon 12 is the most frequently found with a prevalence of ~30% in PanIN neoplasms and nearly 100% of advanced PDAC cases (13-15). In addition, somatic mutations in CDKN2A, TP53 and DPC4/SMAD4 are also observed in patients, although none as frequent as KRAS.

The proto-oncogene KRAS encodes a small guanine nucleotide transferase (GTPase) which cycles between GTP-bound active and GDP-bound inactive states. Thus, while its activation is driven first by the dissociation of GDP followed by the later binding of the GTP

mediated by the guanine nucleotide exchange factors (GEFs), its inactivation is induced by the hydrolysis of GTP therefore returning to the GDP-bound promoted by GTPase-activating proteins (GAPs). Activating mutations of KRAS found in PDAC (point mutations at codon G12, G13 and Q61) impair its intrinsic GTPase activity by blocking the interaction with GAPs leading to its constitutive activation and therefore a persistent stimulation of downstream signalling pathways that drive many of the hallmarks of cancer, sustained proliferation, metabolic reprogramming, anti-apoptosis, remodelling of the tumour microenvironment, evasion of the immune response, cell migration and metastasis (15).

The cell signalling pathways linked to KRAS are considerably complex and dynamic, since involve distinct downstream effectors such as the canonical Raf/Mek/Erk, phosphatidylinositol 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase-1 (Pdk1)/Akt, RalGDS/p38MAPK, Rac and Rho, Rassf1, NF1, p120GAP and PLC-ε (16, 17). However in the context of PDAC it is believed that oncogenic KRAS signalling passes through three main pathways: Raf/Mek/Erk, PI3K/Pdk1/Akt and the Ral guanine nucleotide exchange factor pathway.

The inhibitory crosstalk between the PI3K/AKT and MAPK pathways at the level of AKT and RAF modulates proliferation of the cancer cells (18). This interaction depends on several parameters such as the cell type, the RTK input and even the time course of RTK activation what indeed does not make simple the elucidation of the involved mechanisms (19). Nonetheless Zmajkovicova et al (2013) found a novel crosstalk between PI3K/AKT and MAPK independent of the RTK receptors, which negatively regulates the PI3K signalling by means of the phosphorylation of Mek1 at T292 by Erk resulting in the activation of the complex MAGI1/Mek1/PTEN. Therefore, the inhibition of the MAPK pathway likely interferes PTEN tumour suppressor function leading to PI3K pathway activation. The loss of PTEN and the subsequent activation of the PI3K pathway has been found in PDAC and shown to accelerate tumour formation. This has been attributed to increase the NFκB survival signalling and pro-tumourigenic changes in the tumour microenvironment (20).

The oncogenic KRAS signalling is the main driving force behind PDAC. The signalling networks are characterised by the activation of several effector pathways and these are interconnected at various levels by cross-signalling and feedback loops (Figure 3).

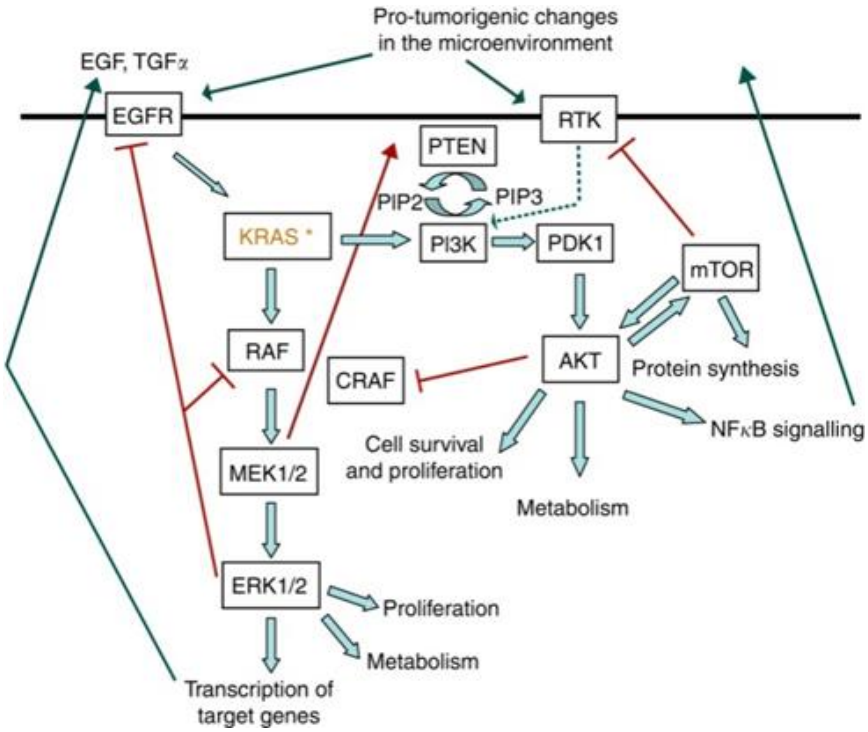

Figure 3. An overview of oncogenic KRAS-driven RAF/MEK/ERK and PI3K/PDK1/AKT signalling networks in pancreatic cancer. (*Eser, S. et al. Oncogenic KRAS signalling in pancreatic cancer. British Journal of Cancer 2014 111, 817–822*).

As it has been aforementioned approximately 5–10% of patients have a family history of the disease (15). Penetrant germline mutations have been found in the tumour suppressors CDKN2A, BRCA2, PALB2, ATM and STK11, the DNA mismatch repair gene MLH1, as well as in the hereditary pancreatitis associated genes PRSS1and SPINK1, and the cystic fibrosis gene CFTR (21-23). Recent genome wide association studies (GWAS) identified multiple loci that harbor common germline susceptibility variants with small effect sizes located in intergenic or intronic regions on chromosomes 1q32.1 (NR5A2) 5p15.33 (TERT/CLPTM1L), 9q34.2 (ABO), 13q22.1 (nongenic) in populations of European descent, and on 3q29 (TFRC), 5p13.1 (DAB2), 6p25.3 (FOXQ1), 7q36.2 (DPP6), 10q26.11 (PRLHR), 12p11.21 (BICD1), 21q21.3 (BACH1), 21q22.3 (TFF1) and 22q13.32 (FAM19A5), in Asian populations (15). Furthermore, mutations

in HNF1A, HNF1B, HNF4A and PDX1 can each cause maturity onset diabetes of the young, and SNPs localizing within the NR5A2, HNF1A, PDX1 and HNF1B gene regions have been identified as markers of pancreatic cancer risk loci through GWAS or pathway based GWAS analyses. Interestingly, unlike it has been reported so far recent transcriptome analysis in PDAC described HNF1A and HNF1B, PDX1 and PTF1A to be among the top five most consistently dysregulated sub-networks (15). Nonetheless, new integrated genomic analysis identified 32 recurrently mutated genes that were aggregated into 10 pathways: KRAS, TGF-β, WNT, NOTCH, ROBO/SLIT signalling, G1/S transition, SWI-SNF, chromatin modification, DNA repair and RNA processing. The subsequent expression analysis defined 4 new subtypes of PDAC: (1) the squamous which are enriched for TP53 and KDM6A mutations, the upregulation of the TP63ΔN transcriptional network together with the hypermethylation of pancreatic endodermal cell-fate determining genes and have a poor prognosis; (2) the pancreatic progenitor that preferentially express FOXA2/3, PDX1 and MNX1 which are involved in early pancreatic development; (3) the immunogenic subtype containing upregulated immune networks including pathways involved in acquired immune suppression; and (4) the aberrantly differentiated endocrine exocrine (ADEX) which displayed the upregulation of genes that regulate networks involved in KRAS activation, exocrine (NR5A2 and RBPJL), and endocrine differentiation (NEUROD1 and NKX2-2 (26, 27).

Studies of Genome-wide analysis of promoter methylation associated with gene expression attributed aberrant gene hyper- and hypo- methylation to contribute to the development and progression of pancreatic cancer (28, 29). Consistently, aberrant methylation increases during neoplastic development among the PanINs and IPMNs (29, 30). Among the list of 1206 candidate BAI1, KCNV1, EYA4, BNC1, HOXA5, PAX7, SOX14, TLX3, NRXN1, CNTNAP2, PKP1, ACTA1, MDFI, EVC2, LIN28, NRN1, PENK, m FAM84A, and ZNF415 were found to be hypermethylated. Whereas within the differential methylation profiles of genes whose epigenetic was silenced included NPTX2, CLDN5, LHX1, WNT7A, FOXE1, PAX6, BNIP3, GADD45B, HIC1, HS3ST2, TWIST1, IRF7, CCNA1, ALPP, CEBPA, CACNA1G, CCND2, and TFPI-2. Interestingly, DNA hypermethylation of genes involved in stem cell

pluripotency, such as the intestinal stem cell marker and chromatin structure regulator BMI1, the genes encoding bone morphogenetic proteins BMP3, BMP6, the transcription factors FOXD3, CDX2, UTF1, and T, as well as NR5A1, NR5A2, NR2F1, NTRK1, NTRK2, NTRK3, NODAL, SALL4, and SPHK1 genes were also identified (31).

Overall, the identification of the accumulated somatic mutations, the aberrantly hypermethylated and silenced genes with the elucidation of the epigenetic mechanisms are crucial for the improvement of diagnosis, prognostic and therapeutic applications.

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Chapter 2

Cancer Stem Cells and Tumour Microenvironment

Cancer Stem Cells

The distinctive characteristic of cancer is the heterogeneity. The major evidence is the diversity present in the subpopulations of the neoplastic cells within the same tumours which strongly describes the co-existence of cancer cells in a multiple levels of differentiation. The diversification give rise to different functional properties throughout the cancer progression. This might be due to the genome instability along with the epigenetic modifications. The heterogeneity may be defined as the intratumoral heterogeneity and intertumoral heterogeneity referring to the molecular differences conferred by tumours initiated in the same organ which allows the classification of these tumours and even representing biologically distinct disease entities. Thus, in the context of the intratumoral heterogeneity the categories that are comprehended are at the level of functionality, phenotype and indeed genetically. Consequently, distinct members co-exist and interact within the same cohort of cells and these interactions may represent one of the major causes responsible of modulating their nature (1, 2).

A unique subpopulation of cells that possess stem properties such as self-renew but also, are able to give rise a diverse progeny with self-limited proliferative capacity thereby perpetuating the malignant growth and even promoting the invasion towards new tissues, receive the name of cancer stem cells (CSC) and their characteristics place them at the apex of an organized hierarchical system within the tumour in where CSC are responsible for the new generation of more differentiated progenies (non-CSC) (1-3).

The origin of the CSC is a question that remains to be elucidated and several studies are emerging in order to address this point. Different origins have been hypothesized however the controversy is mainly serve in a dichotomy manner between the clonal evolution and the CSC paradigm model. The popular theory of Peter Nowell described almost four decades ago about the clonal evolution wherein a single cell undergoes to a sequential cloning leading up to the tumour progression by means of the selection of the most aggressive subclones due to the stepwise acquisition of mutations, has been preserved until nowadays (Figure 1).

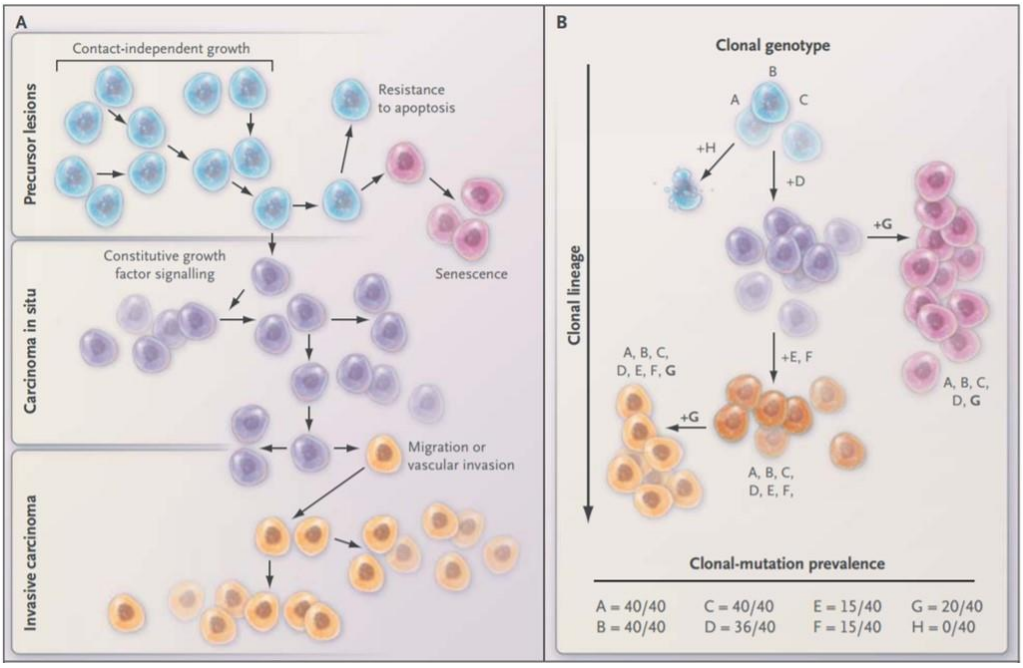

Figure 1. Clonal Evolution and Clonal Relationships. As shown in Panel A, selection operates on the phenotypes termed the "hallmarks" of cancer, giving rise to clones of cells with different properties. Not all properties lead to expansions — for example, increased senescence may lead to eventual stasis or loss of a clone. The acquisition of somatic mutations and alterations in the epigenome (indicated by arrows) can be phenotypically neutral or lead to the acquisition of new phenotypes (cells shaded with distinct colors). As shown in Panel B, progressive accumulation of mutations or other heritable properties leads to clonal relationships. Letters denote a mutation or heritable mark. Colors represent distinct groupings of cells related by descent (clones). Many relationships are possible — for example, mutation G co-occurs independently, leading to two distinct clonal genotypes containing G. The presence of mutations does not necessarily imply selection; the tree depicted could arise owing to random genetic drift. Normal or supporting cells are omitted from this schematic *(Samuel Aparicio, B.M. et al. The Implications of Clonal Genome Evolution for Cancer Medicine. N Engl J Med 2013; 368:842-851).*

In contrast, the CSC paradigm theorize that within a tumour cells only an small and exclusive subpopulation possess self-renewal ability and have the potential to give rise to a variety of transit amplifying cells that divide a certain number of times then differentiate into specialized tumour cells and that unlike their progenitors display limited proliferation and tumorigenicity. Thereby CSC paradigm suggests that tumours may grow like normal tissues of the body.

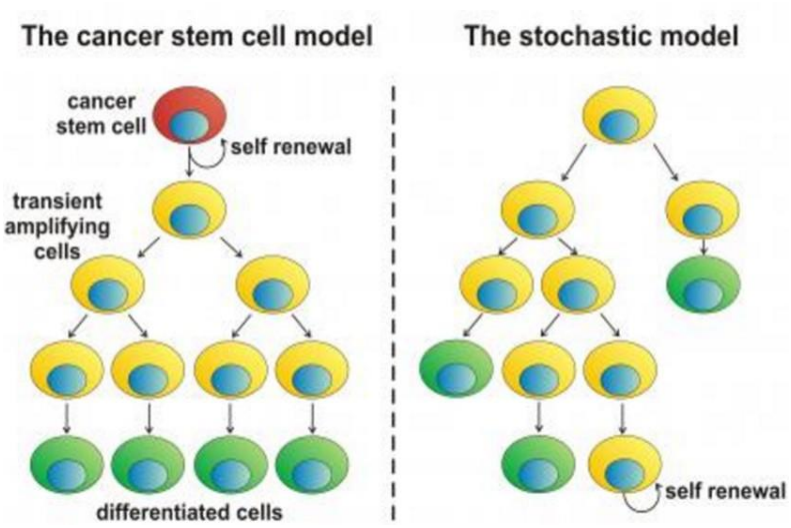

Figure 2. Cancer theories: The cancer stem cell theory suggests a clear hierarchy of cells within a tumour. The stochastic model says that tumour growth is a random process to which all cells can contribute. (*Blanpain, C. et al. EuroStemCell. 2015*).

According to this, the cells within the tumour are in a strictly organised system wherein CSC are at the top of the hierarchy giving rise to more differentiated cancer cells.

On the other hand, inside the CSC paradigm has been proposed a stochastic model of cancer growth. This model explains the tumour growth from different and new approach. This theory argues that all cancer cells have the same potential to grow and divide but each cell is attributed randomly between self-renewal and differentiation. The cells in a tumour are not in an organised system – any cell has the same intrinsic potential to contribute to tumour growth. In fact, different types of cancer may work in different ways, so it is possible that both of these theories are right. Perhaps they apply to different cancers or different stages of tumour development (Figure 2).

The clonal evolution and the two CSC paradigm models are not mutually exclusive, the difficulties in finding clear evidences to discern the main origin are notorious and recent reports suggest that even might exist differences in the origin depending on the type of disease (4-6).

However, our recent data provided new insights that will change the current picture of the CSC paradigm and the clonal evolution theories.

Tumour Cell Plasticity

The tumour cell plasticity comprised the possibility that a non-CSC undergoes to differentiation which ends up turning into a cancer cell endowed of stem-like properties. This bidirectional transformation from non-CSC to CSC has been found in different tumours such as glioma, intestinal tumours and certain types of breast cancers, wherein the common mechanisms are the dysregulation of specific genetic pathways which dictates the emergence and phenotype of the disease.

The process of dedifferentiation refers to the loss of mature functional features and the subsequent possible re-acquisition of the embryonic properties. This concept has been controversial but latest reports reinforcing its existence suggested that could be involved in the transition for tumorigenesis. Thus, the reversion of the differentiation to a stem cell phenotype may suppose an oncogenic transformation.

Despite the apparent differences, every time more the importance of the balance of the tissue homeostasis is becoming more relevant and appears to be the common fact throughout all cancer types. Thus the consequences because of the changes in the tissue homeostasis, known as a tumour microenvironment, exert a major influence in the cell fate and may be decisive for the occurrence of the disease (1-6).

Tumour Microenvironment (TME)

It has been appreciated for some time that the tumour microenvironment (TME) plays a significant role in disease progression, but the precise function of each constituent remains unknown. The TME is constitute by stroma cells and the essential elements that are found to be

part of include fibroblasts, myofibroblasts, neuroendocrine cells, adipose cells, immune and inflammatory cells, the blood and lymphatic vascular networks, and the extracellular matrix (ECM). The stroma is critical for maintaining the physiological normal tissue homeostasis and recent studies strengthened the concept that some stromal components have anticancer activities by regulating immunosuppression and restraining carcinogenesis. Contrary to the stipulated regular conditions the variety of infiltrating immune cells, cancer-associated fibroblasts (CAF) and angiogenic endothelial cells play expanding and critical functions in sustaining cell proliferation, evading growth suppressors, promoting survival, activating invasion and metastasis, and reprogramming energy metabolism (7, 8).

It has been reported the ability of the cancer cells to regulate or modify the stromal cells favouring the cancerous niche by means of the synthesis of cytokines, chemokines and growth factors which consequently accelerate the progression of disease. This stromal cell plasticity leads to respond rapidly to neoplastic situations and act in concert with the adjacent epithelium in eliciting the emergence of "reactive stroma" (9).

In a solid tumour those cancer cells that are in the invasive front are exposed to the reactive stroma, hypoxia and immune surveillance. Thus the invasive front is rich in CAFs, tumour-associated macrophages (TAMs), myeloid progenitor cells and newly generated blood vessels (10). The invasive fronts rich in CAFs and myofibroblasts produces TME-mediated signalling expressing high levels of extracellular factors including chemokine CXC motif ligand 12 (CXCL12), chemokine CC motif ligand 2 (CCL2), CCL8, and insulin-like growth factor binding protein 7 (IGFBF7) promoting the inflammatory response and recruiting immune cells, and also cytokines such as TNF-α, TGF-β, hedgehog (Hgh), Wnt and Notch that are attributed to the survival and fitness of CSCs (8-11).

TGF-β stimulates the epithelial-mesenchymal transition (EMT) thus the carcinoma cells in the invasive front of tumour lose the cell-to-cell adhesion and apical-basal polarity and gain migratory behaviour. Particularly, in pancreatic cancers the enforced expression of EMT transcription factors confers the gain of stem-like features (12). On the other hand TNF-α which

is a crucial growth factor is expanded and released by TAMs establishing paracrine loops supporting back the cancer cells (9). In addition, TNF-α has been also found to stimulate the accumulation of reactive oxygen species (ROS) as well as promote the nuclear entry of β-catenin activating Wnt/β-catenin pathway in surrounding epithelial cells which regulates the stem cell pluripotency and cell fate decisions (Figure 3) (7, 8).

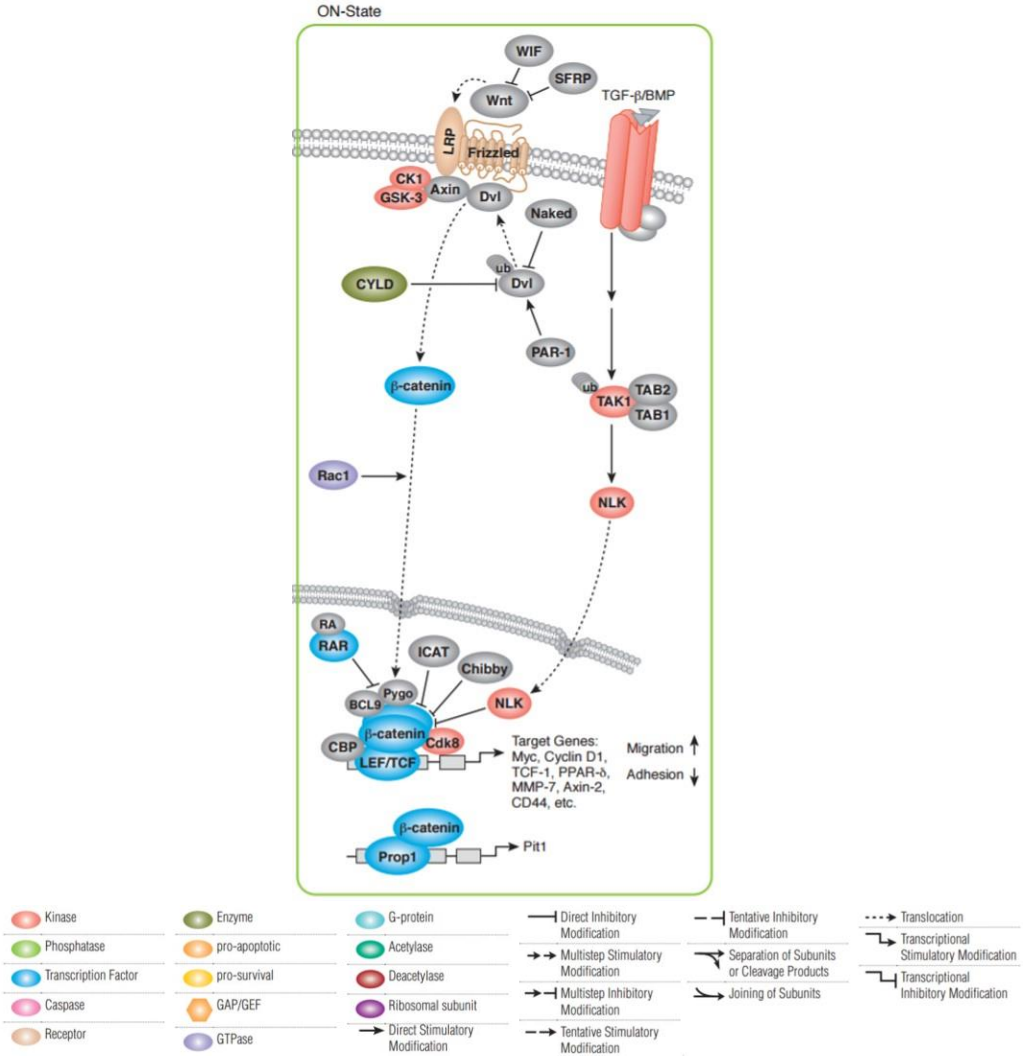

Figure 3. The conserved Wnt/β-Catenin pathway regulates stem cell pluripotency and cell fate decisions during development. This developmental cascade integrates signals from other pathways, including retinoic acid, FGF, TGF-β, and BMP, within different cell types and tissues. The Wnt ligand is a secreted glycoprotein that binds to Frizzled receptors, leading to the formation of a larger cell surface complex with LRP5/6. Frizzleds are ubiquitinated by ZNRF3 and RNF43, whose activity is inhibited by R-spondin binding to LGR5/6. In this manner R-spondins increase sensitivity of cells to the Wnt ligand. Activation of the Wnt receptor complex triggers displacement of the multifunctional kinase GSK-3β from a regulatory APC/Axin/GSK-3β-complex. In the absence of Wnt-signal (Off-state), β-catenin, an integral E-cadherin cell-cell adhesion adaptor protein and transcriptional co-regulator, is targeted by coordinated

phosphorylation by CK1 and the APC/Axin/GSK-3β-complex leading to its ubiquitination and proteasomal degradation through the β-TrCP/Skp pathway. In the presence of Wnt ligand (On-state), the co-receptor LRP5/6 is brought in complex with Wnt-bound Frizzled. This leads to activation of Dishevelled (Dvl) by sequential phosphorylation, poly-ubiquitination, and polymerization, which displaces GSK-3β from APC/Axin through an unclear mechanism that may involve substrate trapping and/ or endosome sequestration. Stablized β-catenin is translocated to the nucleus via Rac1 and other factors, where it binds to LEF/TCF transcription factors, displacing co-repressors and recruiting additional co-activators to Wnt target genes. Additionally, β-catenin cooperates with several other transcription factors to regulate specific targets. Importantly, researchers have found β-catenin point mutations in human tumors that prevent GSK-3β phosphorylation and thus lead to its aberrant accumulation. E-cadherin, APC, R-spondin and Axin mutations have also been documented in tumor samples, underscoring the deregulation of this pathway in cancer. Wnt signaling has also been shown to promote nuclear accumulation of other transcriptional regulator implicated in cancer, such as TAZ and Snail1. Furthermore, GSK-3β is involved in glycogen metabolism and other signaling pathways, which has made its inhibition relevant to diabetes and neurodegenerative disorders. (*Cell Signaling Technology. Created January 2003-revised September 2016.* www.cellsignal.com).

Inflammation and Tumorigenesis

Inflammation has an important role in the tumour initiation. Activated inflammatory cells serve as source of ROS, RNI, growth factors and indeed cytokines that as it has been aforementioned may confer stem-like properties upon the tumour progenitors or even induce the expansion of stem cells (13).

The contribution of myeloid cells to the tumour play a crucial role in promoting tumour progression, angiogenesis, cell invasion and metastasis. Specifically, mast cells are commonly seen in various tumours and have been implicated in the regulation of tumour initiation and development. They are likely the most productive chemical factory in the body and influence other cells through both soluble mediators and cell-to-cell interaction, thereby have the capacity to promote tumour proliferation and invasion both directly by stimulating tumour cells and indirectly by modulating the TME (14). High number of mast cells may be found at the invasive border of tumours. Since they are potent inducers of fibrosis and stimulate fibroblast and myofibroblast proliferation it may lead to the tissue reorganization and the consequent reactive

tumour stroma. In addition, their high source of proteases confer them the ability to degrade the extracellular matrix therefore facilitating the tumour growth and dissemination (15, 16). It has also been seen that the outcome of cancer may come determined by the intricate interaction from mast cells with the regulatory T cells (Treg). TNFα produced by mast cells upregulates OX40 in Treg and activates the PI3K pathway, while MC IL6 works through pg130 to stimulate the JAK–STAT3 pathway, allowing expression of IL17. Proinflammatory properties of Treg may help to propagate tumor growth and dissemination and since mast cells expand in polyps and tumours of the gastrointestinal tract, it is tempting to suggest that mast cells turn the tide in favour of cancer progression by recruiting and then altering the functions of Treg to promote further cancer-associated inflammation. (14). Thus, mast cells appear to have an indispensable role in the development of solid tumours by promoting a gradual gain of pro-inflammatory properties with the participation of Treg cells therefore favouring uncontrolled escalation of cancer inflammation, tumour immune tolerance, and aggressive tumour growth.

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Chapter 3

A new PDAC mouse model originated from iPSCs-converted pancreatic Cancer Stem Cells (CSCcm)

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is the most representative form of pancreatic cancers. PDAC solid tumours are constituted of heterogeneous populations of cells including cancer stem cells (CSCs), differentiated cancer cells, desmoplastic stroma and immune cells. The identification and consequent isolation of pancreatic CSCs facilitated the generation of genetically engineered murine models. Nonetheless, the current models may not be representative for the spontaneous tumour occurrence. In the present study, we show the generation of a novel pancreatic iPSC-converted cancer stem cell lines (CSCcm) as a cutting-edge model for the study of PDAC. The CSCcm lines were achieved only by the influence of pancreatic cancer cell lines conditioned medium and were not subjected to any genetic manipulation. The xenografts tumours from CSCcm lines displayed histopathological features of ADM, PanIN and PDAC lesions. Further molecular characterization from RNA-sequencing analysis highlighted primary culture cell lines (1st CSCcm) as potential candidates to represent the pancreatic CSCs and indicated the establishment of the pancreatic cancer molecular pattern in their subsequent progenies 2nd CSCcm and 3rd CSCcm. In addition, preliminary RNA-seq SNPs analysis showed that the distinct CSCcm lines did not harbour single point mutations for the oncogene Kras codon 12 or 13. Therefore, PDAC-CSCcm model may provide new insights about the actual occurrence of the pancreatic cancer leading to develop different approaches to target CSCs and abrogate the progression of this fatidic disease.

Introduction

PDAC is one of the most highly desmoplastic tumours which unfortunately due to its aggressiveness and rapid dissemination together with the strong resistance to the radiation therapy and chemotherapy contributes to the dismal prognosis. Over the past decades the strategies to find new diagnostic approaches at early stages along with the effective treatments has not improved significantly (1, 2). Therefore, this highlights the urgent need to find novel models to study the origin as well as the progression of the disease.

PDAC solid tumours are comprised of a wide range of heterogeneous populations of cells including cancer stem cells (CSCs), the actual differentiated cancer cells together with desmoplastic stroma and immune cells which represent a high proportion of the tumour mass (3). CSCs are considered as cells that possess stem cell properties and produce diverse lineages of cancer cells. Hence, CSCs have been associated with the tumour initiation and progression, and have been reported to be involved in tumour metastasis (4).

The isolation of pancreatic CSCs succeeded in providing new insights regarding the chemoresistance and the high metastatic ability in PDAC. Since CSCs and non-CSCs share an identical genetic background it is difficult to find appropriate *in vitro* and *in vivo* systems that allows to select reproducibly and exclusively enriched CSCs populations. Furthermore, these new approaches include particular markers that are also found in differentiated adult cells what makes questionable the identification of the CSCs (7). Recent advances have been developed in targeting CSCs and their identification and isolation consequently facilitate the generation of new murine models (5, 6). However, the current models are genetically engineered and therefore may not be suitable for a better understanding of the spontaneous tumour occurrence.

As have been seen in regenerative medicine field, iPSCs when exposed to appropriate environments are able to directly differentiate into progenitor cells that lead to the latter matured form of cells. Hence, the signals found in the niche simultaneously regulate the differentiation as well as support the tissue homeostasis preserving the self-renewal potential from a minor but

required stem cells number (8). Based on this we previously hypothesized that CSCs might be considered as progenitor cells that are destined to differentiate into cancer cells and that consequently if the cell fate comes determined by the events and factors present in the niche, the tumour microenvironment should exert the same effects when healthy cells are exposed to it. Chen L and Kasai T et al. demonstrated the impact of the so-called *cancerous niche* when by exposing Nanog iPSCs to a Lewis Lung carcinoma conditioned medium (LLCcm) a malignant tumour was obtained exhibiting angiogenesis *in vivo*, capacity of self-renewal and expressed markers associated to stem cell properties and undifferentiated state such as Nanog, Rex1, Eras, Esg1 and Cripto. In contrast, when control Nanog iPSCs were implanted into Balb/c nude mice formed typical teratomas displaying contained differentiated tissues without metastasis. Thus a new model of CSC-like cells generated exclusively under the influence of the microenvironment was proposed (9).

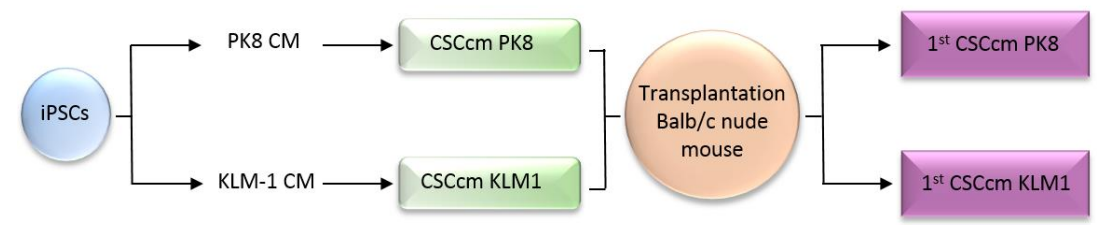
In the present study, we show the generation of a novel pancreatic iPSC-converted cancer stem cell lines (CSCcm) together with the subsequent characterization of the tumours obtained as a result from the transplantation of the CSCcm lines *in vivo* demonstrating that CSCcm is a promising cutting-edge model for the study of PDAC occurrence and progression.

Results

iPSCs-converted CSCs (CSCcm) display CSCs features

As described in the protocol established by Chen L and Kasai T et al., to generate a model of pancreatic CSCs, iPSCs were exposed to different conditioned medium (CM) from pancreatic carcinoma cell lines PK-8CM and KLM-1CM, a process named as *conversion* (Figure 1A) (9). Similarly to the established protocol, iPSCs were maintained in feeder-less conditions and the Leukemia inhibitory factor (LIF) which is essential for their viability was removed from the medium and thereby restricting them to the effect exclusively of the conditioned medium. Whereas the iPSCs cultured only with iPSCs media without LIF expired after 7 to 10 days (Figure 1B), the viability and proliferation of the cells maintained in presence of conditioned medium was not affected. Nanog iPSCs cells were generated by the retrovirus-mediated introduction of the four factors Sox2, Oct3/4, Klf4 and c-Myc into the Nanog-GFP-IRES-Puro^r of mouse embryonic fibroblast (42), therefore iPSCs could be monitored throughout the conversion by the GFP expression as a validation of the stemness. The population of converted cells displayed a diverse pattern of differentiation harbour cells expressing strong to moderate GFP and other differentiated cells which the expression was null. Once the process was completed the cells were termed as CSCcm PK8 and CSCcm KLM-1 and their stem-like properties were tested through sphere suspension assay (Figure 1B).

A



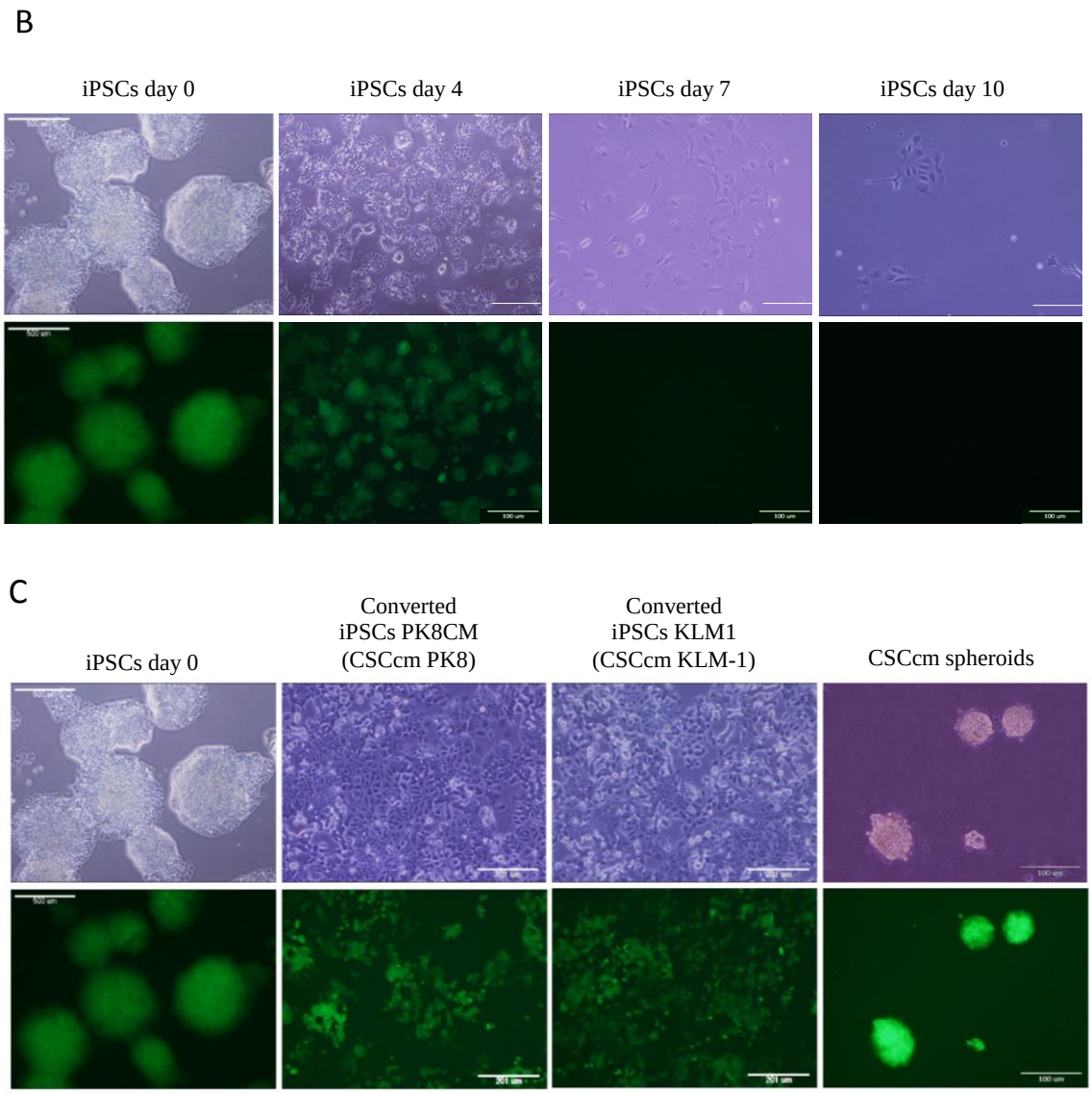


Figure 1. A) Representative scheme of the conversion procedure. B) Viability of iPSCs maintained in control medium without LIF was no longer than 10 days. iPSCs underwent differentiated with no remaining GFP positive cells and eventually expired (upper panel). Stemness tracking during conversion by the presence of GFP protein. Self-renewing potential was validated by sphere formation assay previously to the subcutaneous implantation (lower panel). Original magnification 4x and 20x.

Since the CSCcm population was heterogeneous, in order to ensure a high frequency of cells displaying CSC-like properties, 10^4, 10^5 and 10^6 cells from the two new pancreatic CSCcm lines were subcutaneously transplanted into immunocompromised Balb/c nude mice. After 30 days, CSCcm engrafted and generated tumours in 9 out of 9 mice for each cell line indicating experimental reproducibility and demonstrating their tumorigenic potential. Chen and Kasai et al.

previously shown that iPSCs cultured under control medium generated benign teratoma. Consistently, the xenograft tumours histology showed specific characteristics that resembled the actual pancreatic ductal adenocarcinoma phenotype. Primary tumours were rich in stroma and among the epithelial-like structures pancreatic intraepithelial neoplastic lesions (PanIN) lesions were found together with moderate to poorly differentiated ductal structures (Figure 2A).

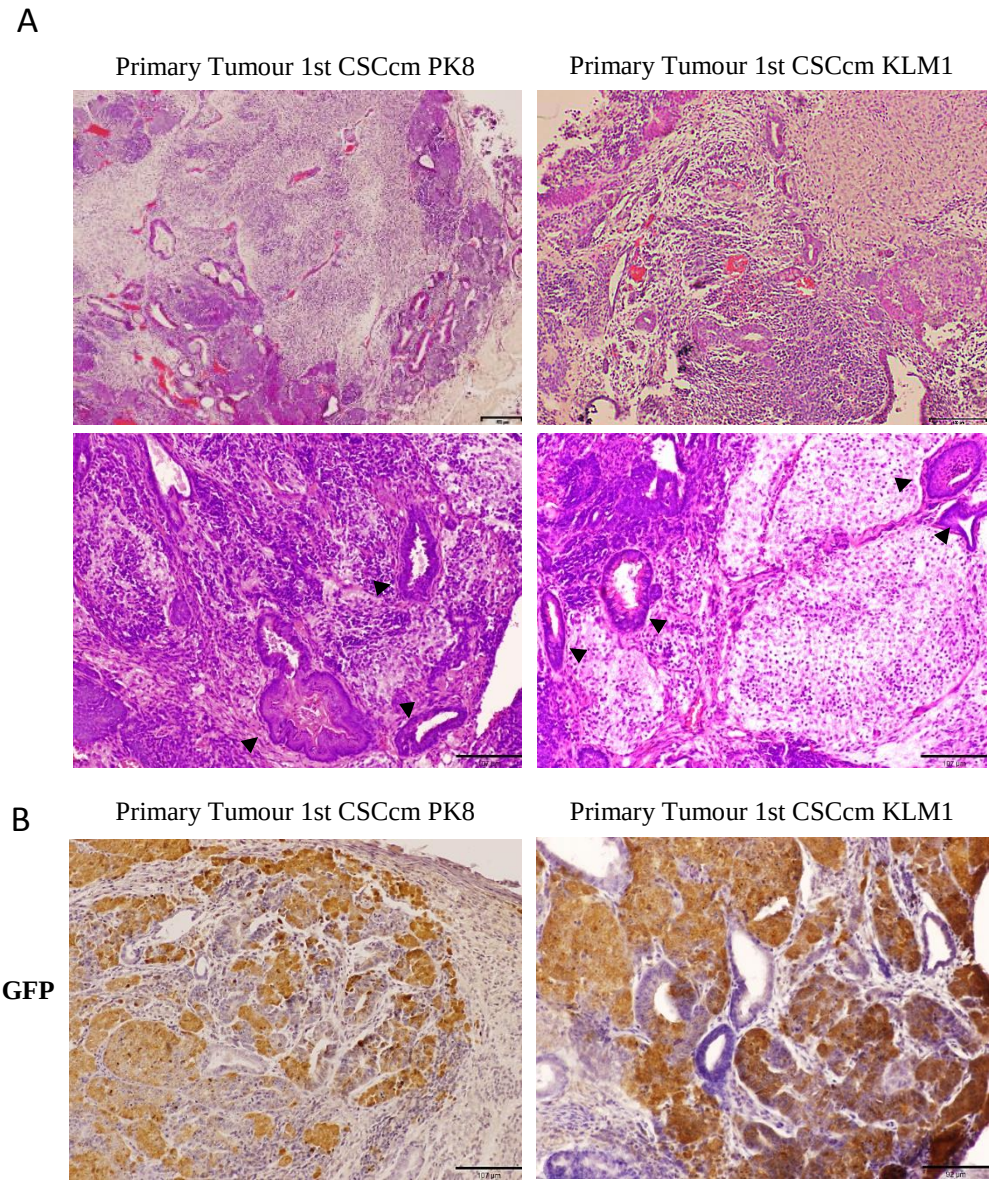
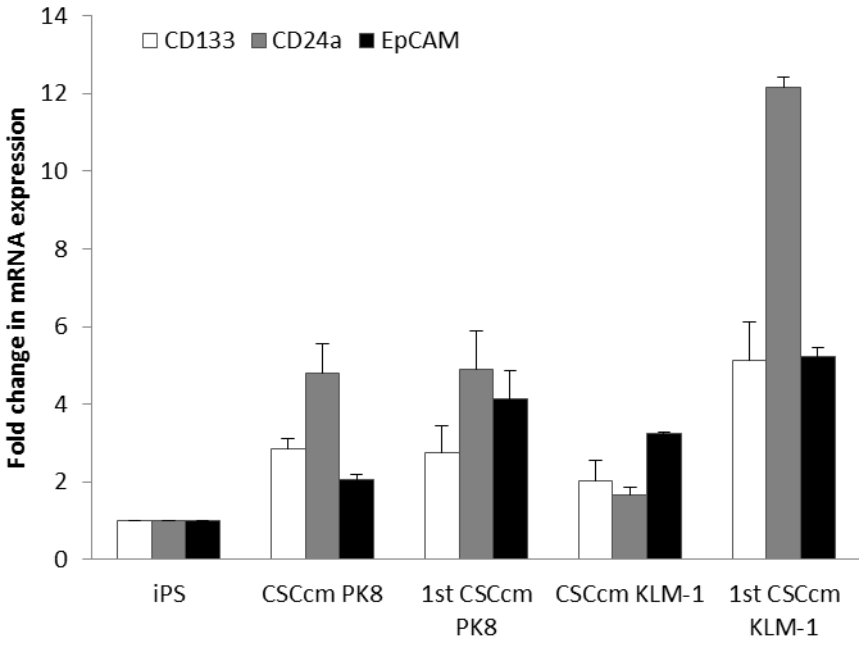


Figure 2. A) Histopathological features of 1st CSCcm primary tumours were evaluated by H&E staining. Specific PanIN lesions are indicated with arrowheads. Original magnification 10x and 20x. B) Lineage tracing by GFP protein showed that it was predominantly expressed in undifferentiated cells, however was also partially found in ductal-like structures. Original Magnification 10x and 20x.

To further validate the PDAC-like structures arose from CSCcm we explored the expression of GFP protein in primary tumour. GFP expressing cells were found all over the tissue samples and although it was predominantly located by undifferentiated cells few epithelial cells from ductal-like structures remained positive for its expression (Figure 2B). When the expression of specific CSC markers CD133, CD24a and EpCAM was evaluated, their up-regulation was already observed in CSCcm and was similarly detected or even enhanced in primary cultures (1st CSCcm) (Figure 3A). CD133 is preferentially used in the identification of CSC in pancreatic solid tumours and its expression is commonly found within the ductal structures. Likewise the primary CSCcm tumour tissues showed a strong signal within the ductal-like structures (Figure 3B) confirming their malignant phenotype.

A



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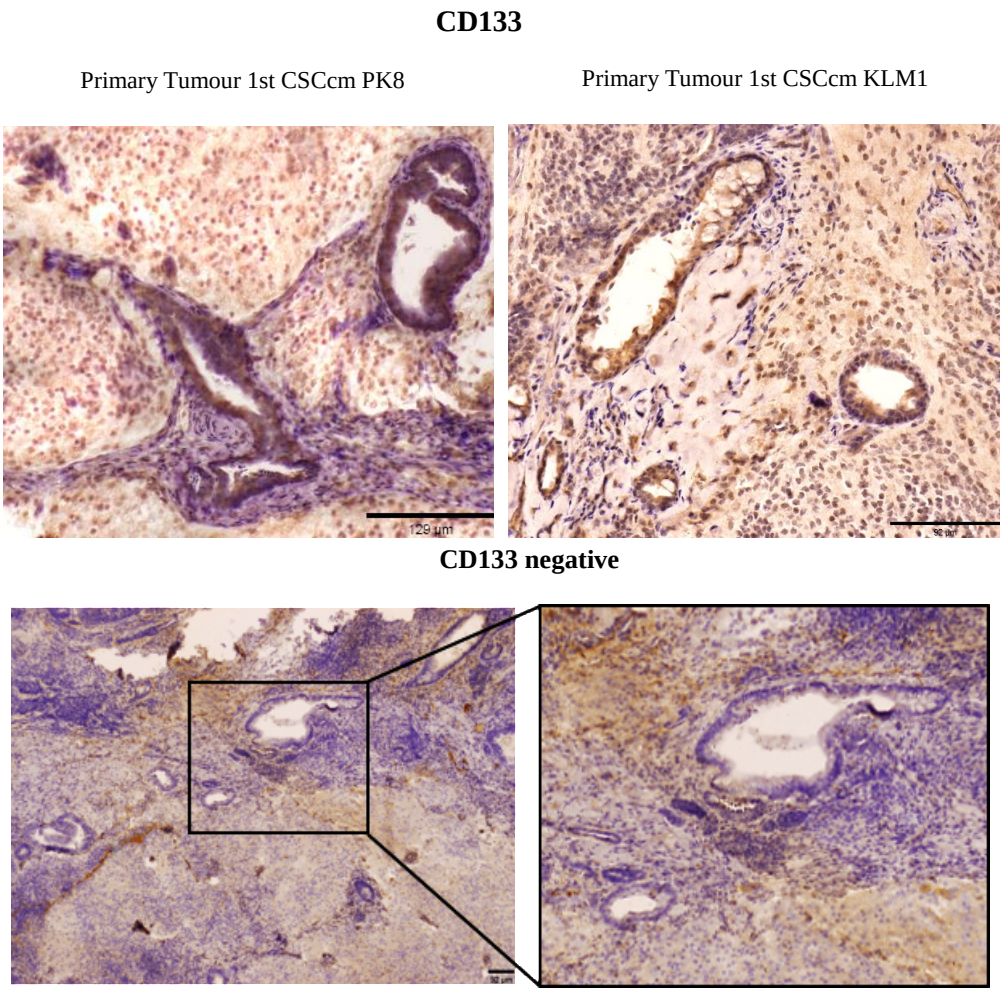


Figure 3. A) RT-qPCR analysis of CSCs markers CD133, CD24a and EpCAM in converted cells CSCcm and primary cultures 1st CSCcm. B) Upper micrographs showing the CD133-satined tissues from 1st CSCcm tumour. Positive expression was mainly located in ductal cells. Negative control for CD133 expression is shown in lower panel. Original magnification 15x and 10x.

Primary cultures 1st CSCcm were enriched by puromycin allows the possibility to select the lineage from the top level of hierarchical system obtaining the corresponding lineage from the top of the cell hierarchy system and avoiding the intrusion of cells from the host. As compared to CSCcm it was noteworthy that 1stCSCcm generated a specific cell distribution composed by well-defined colonies surrounded by myofibroblast-like cells most likely PSCs (pancreatic stellate cells). This particular feature of generating a spontaneous *self-supporting* system for the integrity and maintenance of the 1st CSCcm population was not observed neither in iPSCs control nor

CSCcm cultures, therefore might be considered as an indicative of their enhanced malignant transformation (Figure 4).

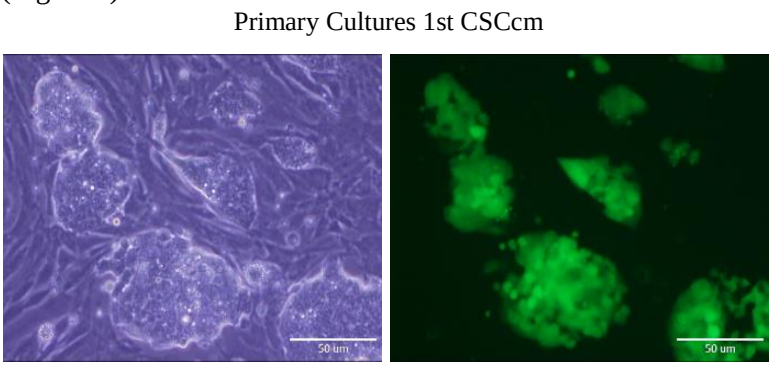


Figure 4. Cell distribution of primary cultures (1st CSCcm) after puromycin enrichment.

Serial Transplantation leads to a more established PDAC phenotype

With the purpose to assess whether CSCcm display long-term tumorigenic potential we performed serial transplantation. Thus, cells from 1st CSCcm were transplanted into secondary and subsequently into a tertiary nude mouse (Figure 5).

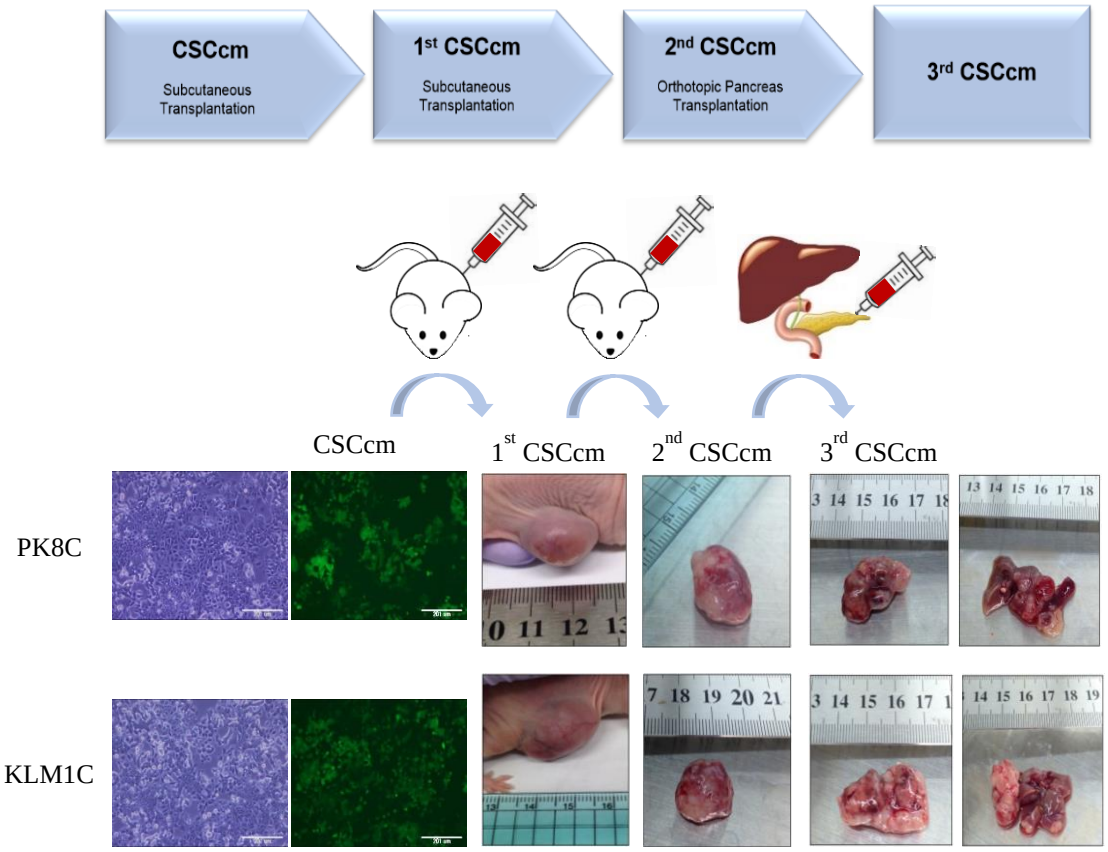


Figure 5. Serial transplantation scheme and images from sequential excised tumours obtained from subcutaneously transplanted CSCcm and 1st CSCcm primary cultures. Primary tumours and their corresponding metastatic liver nodes generated from orthotopically implanted 2rd CSCcm cells are also shown. Experiments were equally performed for both PK8CM and KLM1CM lines.

Similarly to the primary culture the cells from secondary culture (2ndCSCcm) as well as tertiary cultures (3rdCSCcm) were enriched by puromycin. Thereby 1stCSCcm PK8 and 1stCSCcm KLM-1 were subcutaneously transplanted giving rise tumours within a short period of time of 25days. Next, 2ndCSCcm PK8 and 2ndCSCcm KLM-1 were subsequently transplanted, however this time cells were orthotopically transplanted into the pancreas generating tumours within 15-20days. Orthotopic tumours were remarkably bigger and specific liver metastasis was found (Table 1).

Table 1. Primary CSCcm tumours from PK8CM cell lines and KLM-1CM cell lines obtained after the serial transplantation.

Primary Tumours	**Time (days)**	**Volume (mm^3)**	**Metastasis**
1st CSCcm PK8	30	2300	No
2nd CSCcm PK8	25	2600	No
3rd CSCcm PK8	20	3000	Liver
1st CSCcm KLM1	30	2500	No
2nd CSCcm KLM1	28	2100	No
3rd CSCcm KLM1	18	3000	Liver

Interestingly, subcutaneously transplanted tumours displayed indistinguishable histopathological morphologies whereas orthotopic primary tumours were richer in stroma mostly located in the inner mass and less epithelial ductal-like structures were found at the edges of the tumour. This may likely be due to difference in differentiation time whereby the myofibroblast-

like phenotype is acquired earlier the epithelial-like phenotype requires a longer period of time (Figure 6).

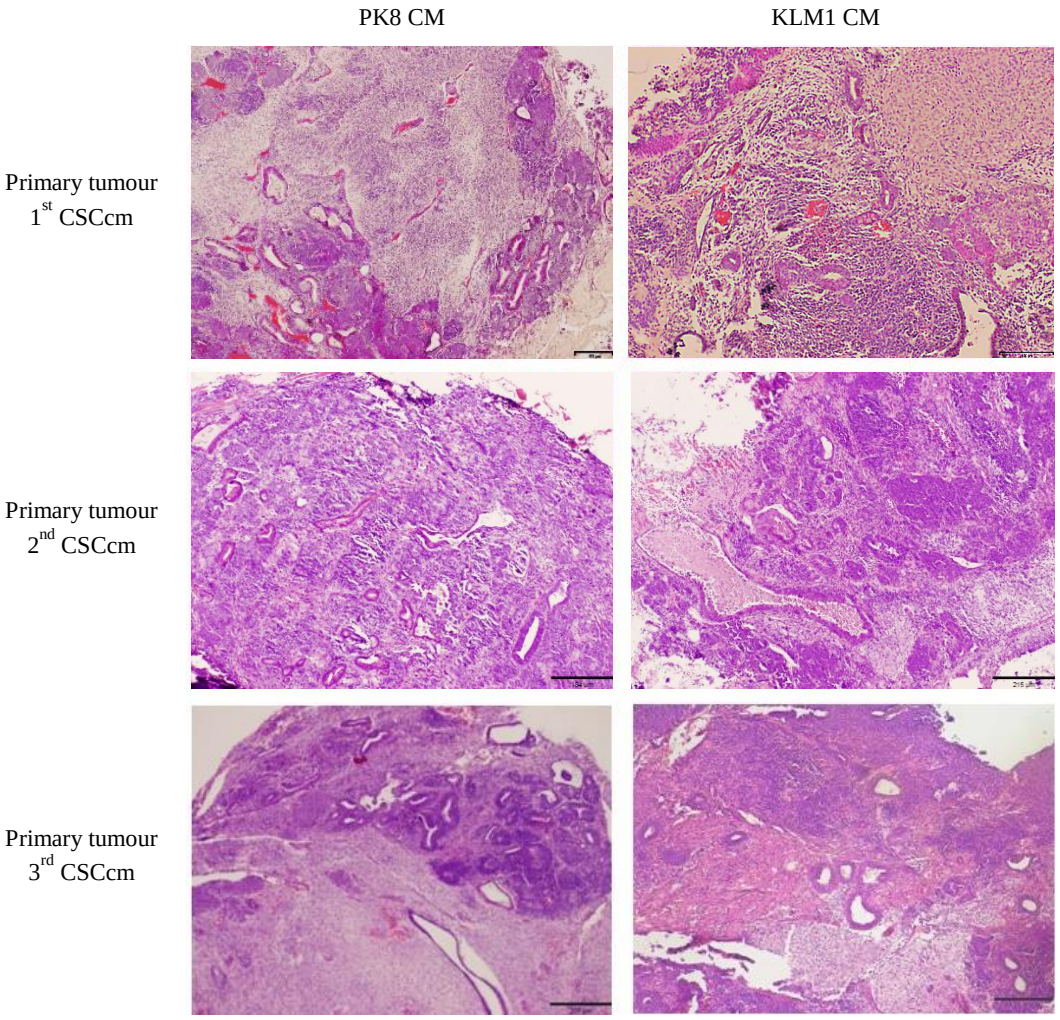


Figure 6. Micrographs from serial primary tumours showing the histopathological adenocarcinoma-like morphology by H&E staining. Original magnification 4x.

When the liver metastatic nodes were examined a remarkable difference in the histopathological features was observed. Unlike primary tumours, liver metastatic nodes displayed a teratocarcinoma phenotype containing very few structures corresponding to PDAC. To determine that CSCcm had the ability to metastasise we searched evidences of GFP protein.

Its expression was strongly localised in areas with undifferentiated embryonic-like cells and no evidences were seen in more differentiated regions (Figure 7).

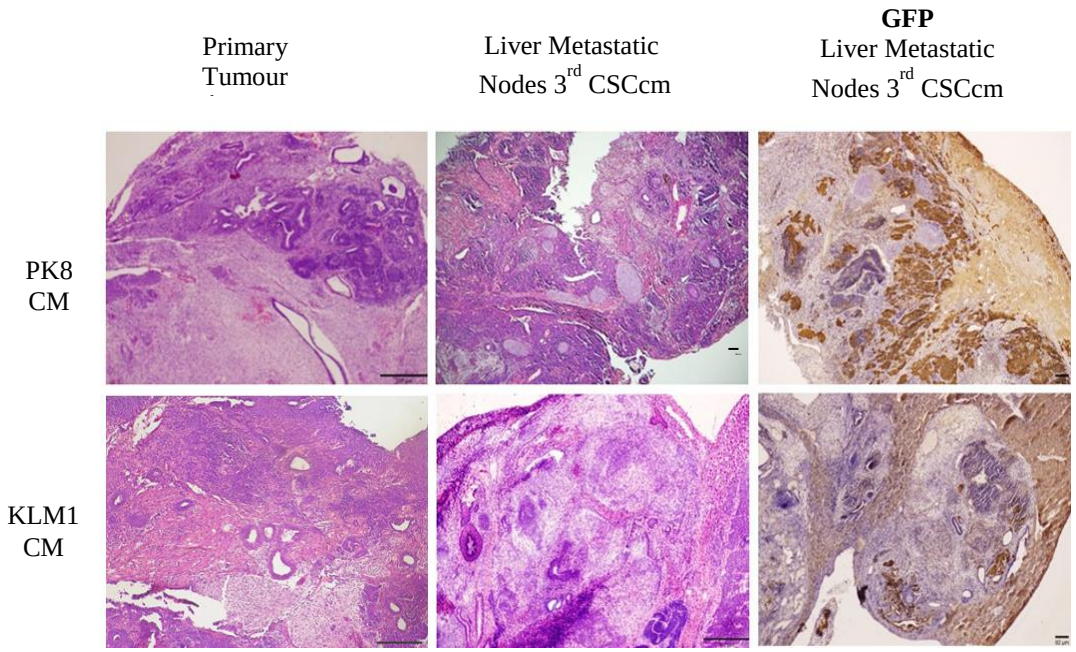


Figure 7. Comparison of the histopathological features between 3rd CSCcm primary tumours and 3rd CSCcm liver nodes. 3rd CSCcm primary tumours displayed adenocarcinoma-like phenotype while a clear teratocarcinoma structure was found in liver nodes. GFP protein expression was shown to be located in undifferentiated cells areas within the liver metastatic nodes. Original magnification 10x.

This difference made us question the integrity of CSCcm and whether a possible reversion towards the iPSCs phenotype was occurring. Nonetheless, complementary lines of investigation by our group reported tumours harbouring adenocarcinoma phenotype when transplantations were held into mammary gland and confirmed the teratocarcinoma phenotype when transplanted directly into the liver most likely because of the strong endocrine potential that liver microenvironment owns (data not shown). Thus, determining the major influence that the microenvironment exerts on the CSCcm fate.

Given that the reprogramming of iPSCs was performed with the proto-oncogenes Sox2, Oct3/4, Klf4 and c-Myc, and that the implication specifically of c-Myc and Klf4 have been linked to PDAC we explored the expression of the transgenes in order to determine that there was no residual activity (Figure 8A). On the other hand the transcript levels of endogenous c-Myc, which is generally overexpressed in PDAC, were notably increased (Figure 8B). Hence, strongly supported that the malignant transformation arose by means of the intrinsically activated mechanisms of the cells.

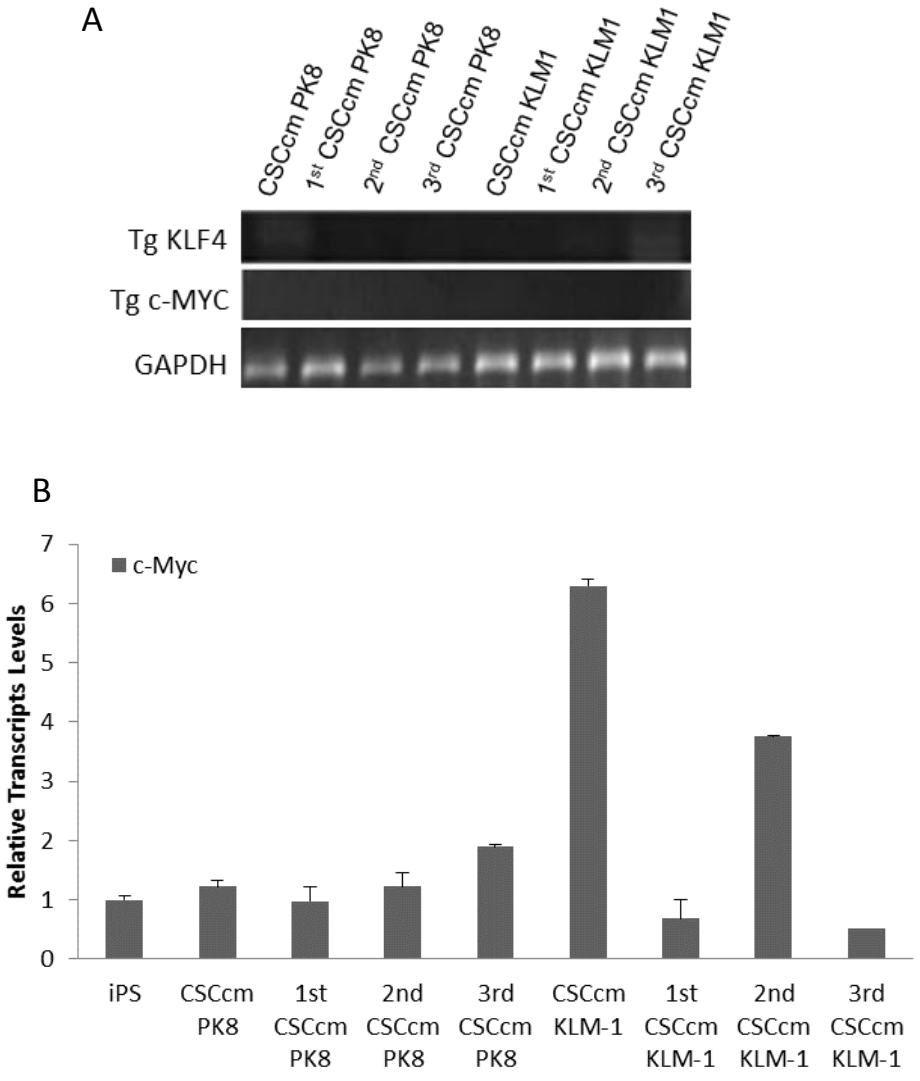


Figure 8. A) Agarose gel images from RT-qPCR products for the detection of Klf4 and cMyc transgenes. GAPDH was taken as a housekeeping control gene. B) Transcript levels for the mRNA of endogenous c-Myc were analysed by RT-qPCR in a panel of serial transplantation samples.

Even though the resulting CSCcm populations from the serial transplantation gave rise to more aggressive phenotype, with regard to the assessment of pancreatic CSC markers intriguingly a decreased expression was observed in 2ndCSCcm and 3rdCSCcm suggesting a possible establishment of the lineage that results into more differentiated pancreatic cancer cells (Figure 9). This is later discussed by RNA-seq DEG analysis that demonstrated an up-regulation of the pancreatic cancer cell hallmarks (Figure 6C).

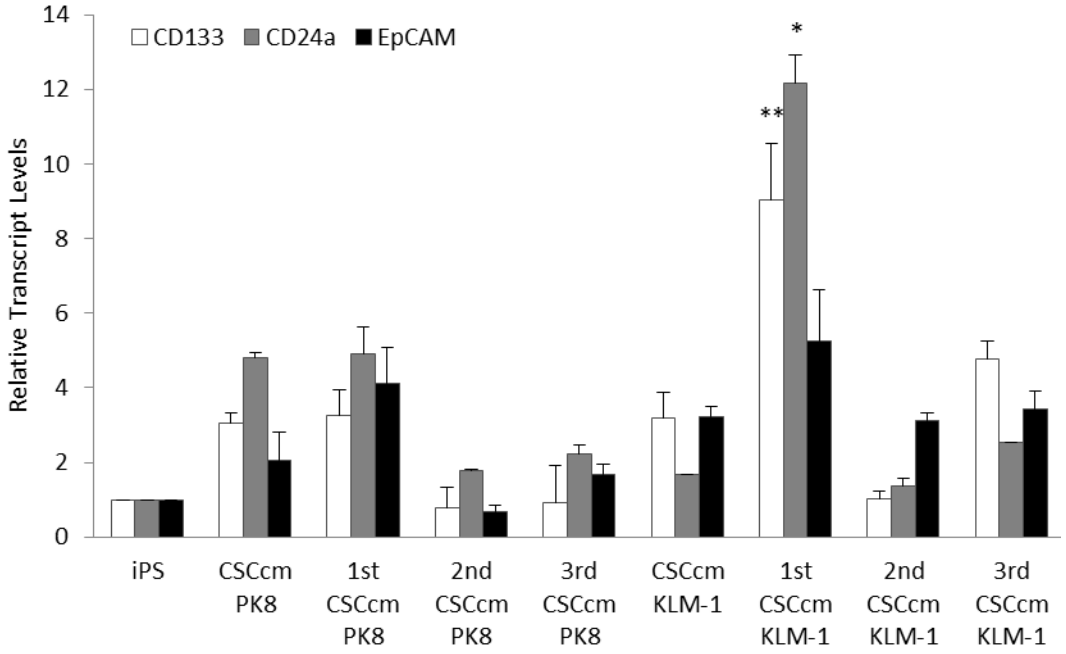


Figure 9. RT-qPCR analysis of the preferentially expressed CSCs markers in a panel of serial transplantation samples.

Characterization of CSCcm lines and primary tumours by PDAC hallmarks

Bailey P. et al. (March 2016) presented a new definition for 4 pancreatic cancer subtypes based on an integrated genomic analysis. Among these 4 different subtypes is found the pancreatic progenitor class wherein the main transcriptional networks are comprised by transcription factors involved during the embryonic pancreatic differentiation (10). In order to assess PDAC characteristics in the serial CSCcm lines, we hypothesise that CSCcm populations most likely may share similar molecular pattern as found in the pancreatic progenitor subtypes. Within the main transcription factors described by Bailey P. et al, PDX1, FOXA2 and HES1 were cited to

be essentially expressed in the progenitor subtype. Thus, their transcript levels were examined along with the KRAS which is well-known to be involved in PDAC. Despite the variation found between the populations most likely due to the uncontrollable fate of the lineage, the up-regulation was apparent for all pancreatic progenitor markers as well as it was for KRAS (Figure 10).

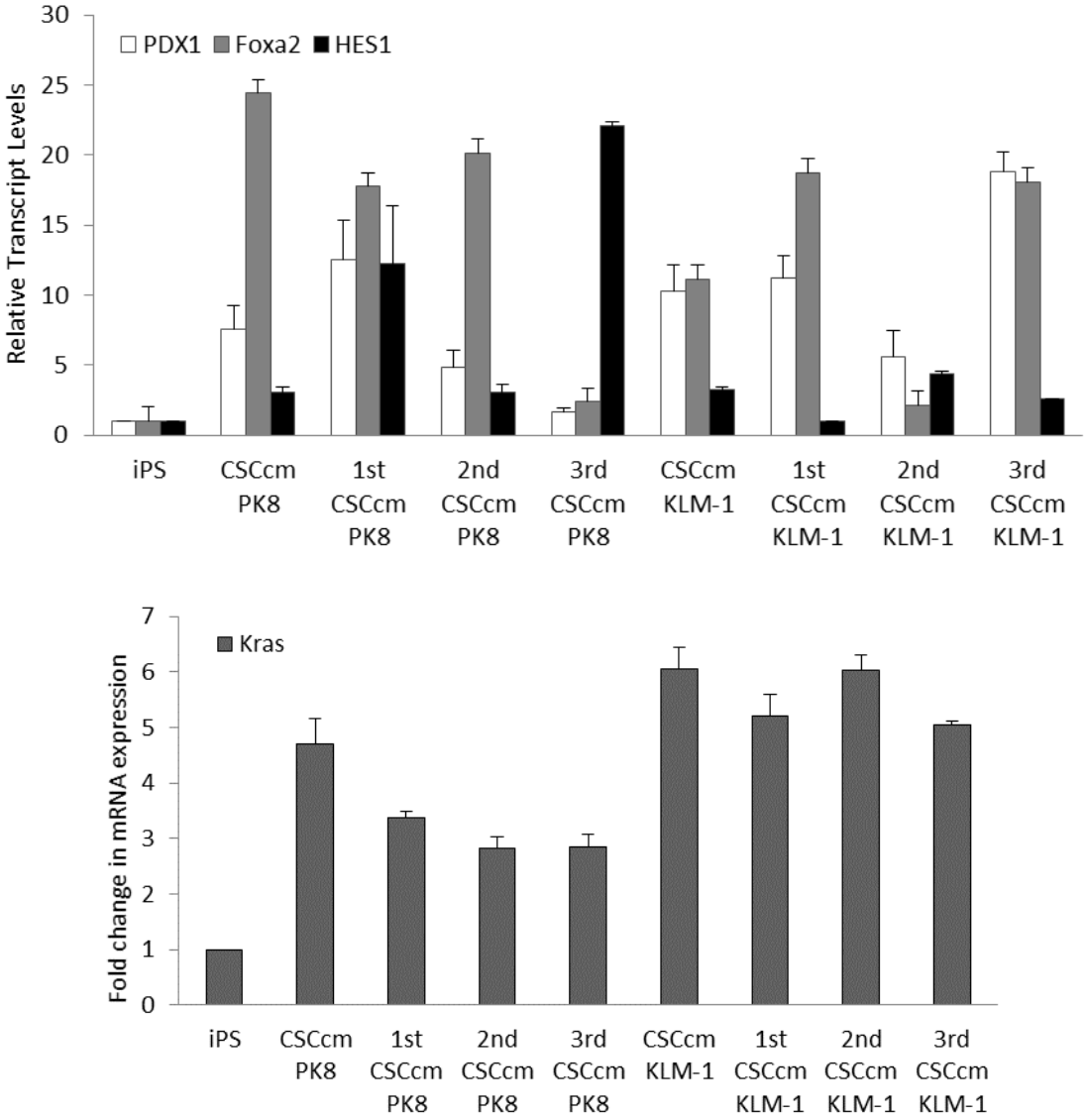


Figure 10. Pancreatic progenitor markers Pdx1, Foxa2 and Hes1 mRNA transcript levels and Kras mRNA expression levels were analysed through RT-qPCR analysis in a panel of all serial transplantation samples.

Likewise to the described pancreatic progenitor class, the adenocarcinoma markers MUC1 and MUC5aC were co-expressed in all CSCcm tumours samples (Figure 11) specifically located at the membrane of epithelial ductal cells. Interestingly few structures that presented

ductal ectasia displayed strong expression in the infiltrating immune cells. Therefore these evidences infer in the achievement of CSCcm lines to recapitulate the PDAC phenotype.

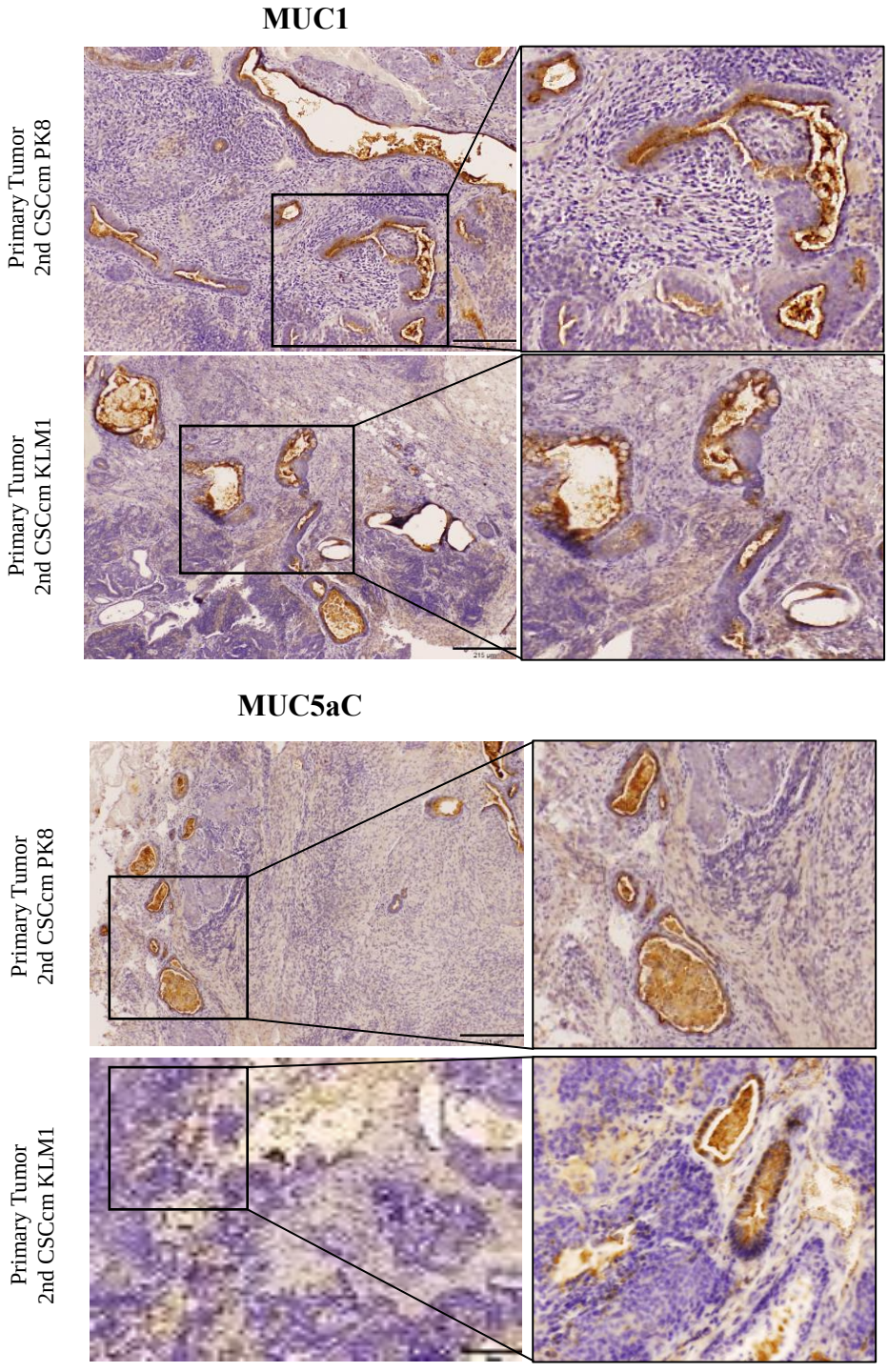


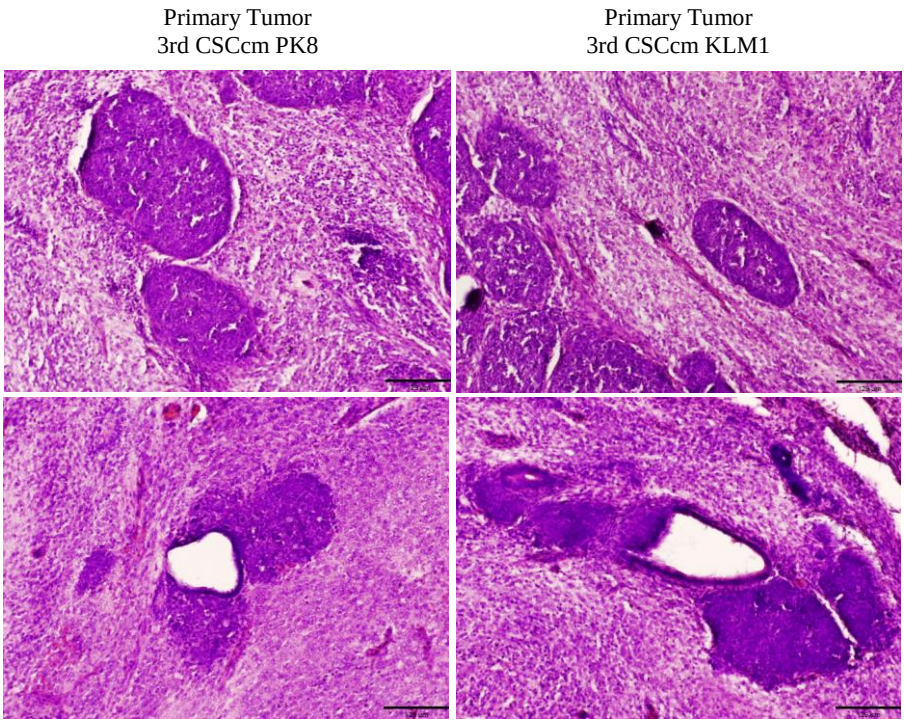
Figure 11. The adenocarcinoma phenotype was evaluated through the expression of the apomucinous of MUC1 and MUC5aC expression was predominantly located in the membrane of ductal structures. Infiltrating ductal immune cells also displayed high expression was also observed in mucinous ductal ectasia (MDE). Original magnification 4x.

A possible model for lineage tracing ADM events

The acinoductal metaplasia (ADM) describes the process whereby the islet neogenesis is accompanied by the transdifferentiation of the normal exocrine tissue into ductal complexes. This pancreatic metaplasia may turn into a premalignant state by means of progressive changes in the ductal epithelium that gives rise to PanIN lesions and may eventually lead to the progression of PDAC (11, 12). Thus, proposed linear progression models for PDAC where ductal cells evolve into hyperplastic and later into dysplastic epithelium resulting in an invasive carcinoma had to be reconsidered since there are no formal evidences of these sequential events. Instead, *in vitro* and genetically engineered mouse models of PDAC have shown that tumours can arise from acinar cells (7).

When orthotopically transplanted 3rdCSCcm tumours were examined in detail large regions composed of highly desmoplastic stroma could be seen surrounding clusters of cells that resembled the acini morphology. Moreover, multiple ducts were also found to co-localize with the acinar-like areas and few of them showed evidences of ADM transition (Figure 12A-B).

A



B

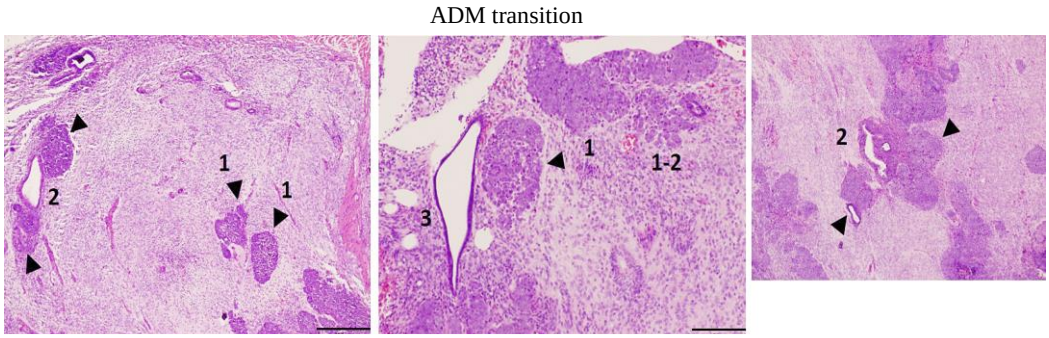


Figure 12. A) H&E staining of cluster cells areas with acinar morphology and ADM structures found in 3rd CSCcm primary tumours. B) Images showing evidences of ADM transition originated from acinar structures (arrowheads) indicated as 1, acino-ductal interphase as 2 and final ductal-like acquired phenotype 3.

With the purpose to determine the acinar phenotype the presence of the protein Ptf1a was assessed. The resulting Ptf1a expression was specifically located in the cell clusters demonstrating a primary level of acinar differentiation. In addition, few PanIN lesions together with well-differentiated ductal structures were found nearby the acinar-like cells and the intermediate stages of ADM. The expression of GFP was found to co-localized in the Ptf1a positive cell clusters validating the acinar cells as the lineage of CSCcm (Figure 13).

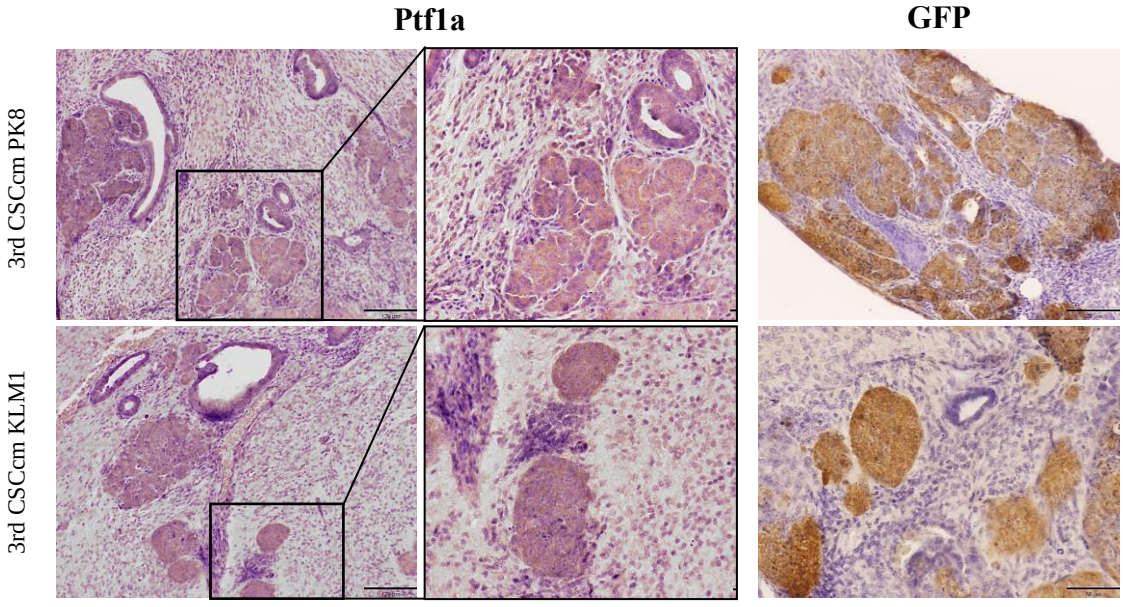
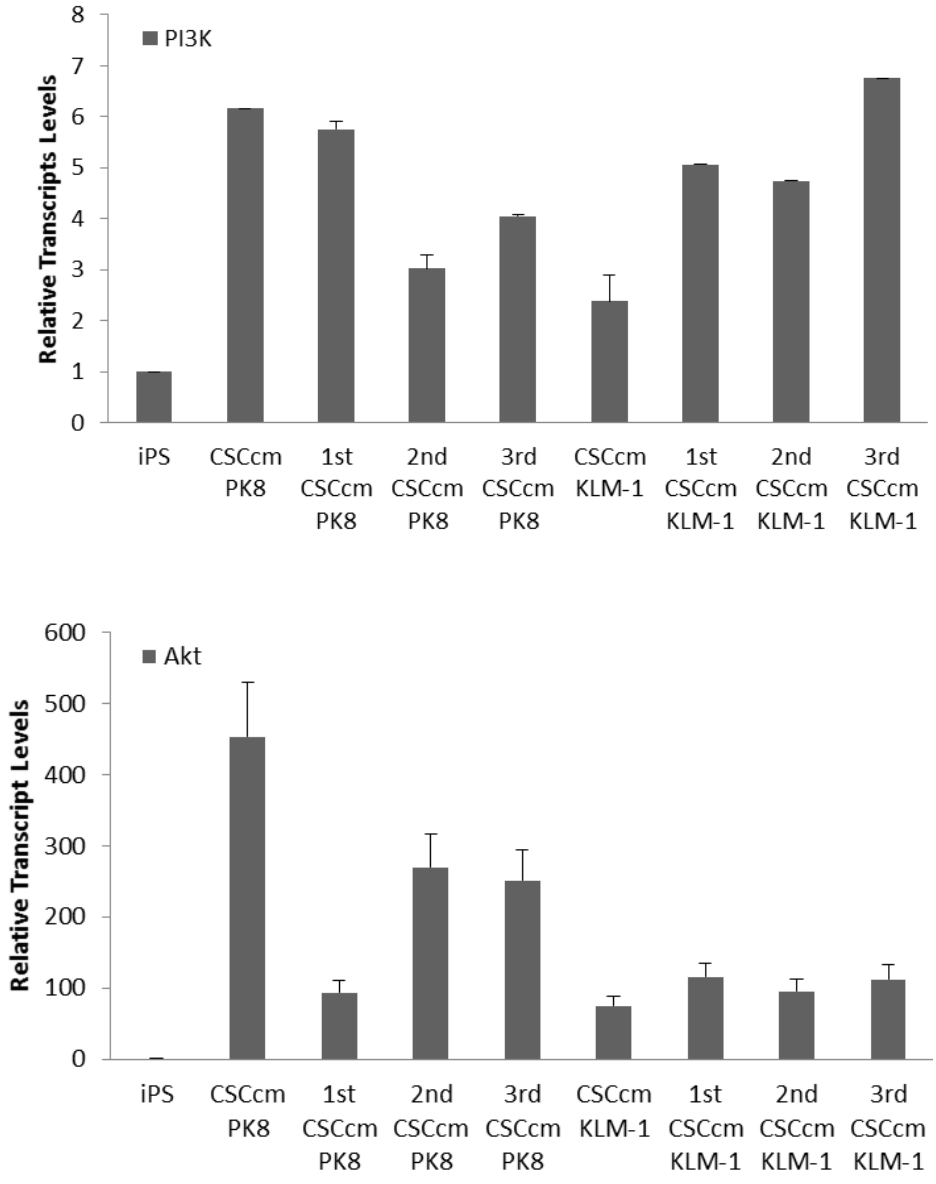


Figure 13. Cell clusters and few ductal cells were positive for the acinar marker Ptf1a. GFP was equally predominantly located in cell clusters and few cells from ductal epithelial cells. Micrographs original magnification 10x and 20x

Supporting these evidences we detected the overexpression of Akt which through the PI3K/Akt pathway have been reported to be involved in the acinar dedifferentiation along with the accumulation of the β-catenin in the cytoplasm that has also been associated (Figure 14) (13-16). Since it is important to elucidate whether the process of dedifferentiation plays an important role in the appearance of early events in pancreatic cancer our CSCcm model may be suitable for lineage tracing how acinar cells undergo into dedifferentiation losing its mature features and discern whether eventually develop a malignant transformation.



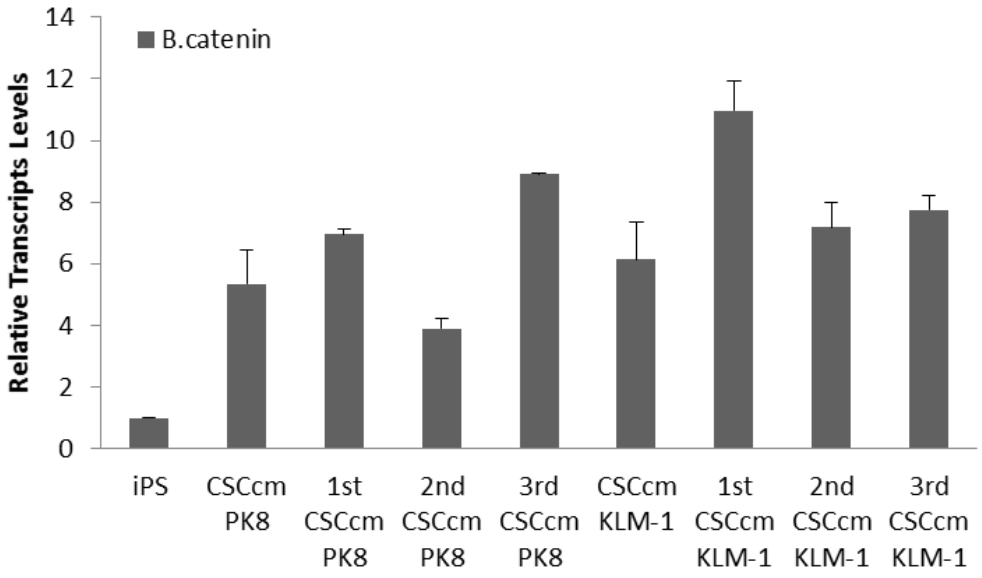


Figure 14. RT-qPCR of the relative transcript levels for PI3K, AKT and β-catenin in a panel of the samples obtained from serial transplantation.

Molecular characterization confirms the acquisition of CSC features and a subsequent established PDAC pattern

To further validate the molecular nature of the CSCcm from PK8CM lines a transcriptome analysis by RNA-sequencing was performed. The expression quantification of 35,276 total described genes was estimated by Fragments Per Kilobase Million (FPKM) [$\log_{10}$(FPKM+1) value] and members from the transcriptional pancreatic progenitor network Pdx1, Hes1, Foxa2, Hnf1a, Hnf4a, Pax6, Nr5a2, Rbpj, Rbpgl, MafA and MafB were found to be expressed together with PDAC related hallmarks such as KRAS, Krt19, Col8a1, Col1a1, CXCR4, Muc1, Muc5aC, Mmp2 or MALAT1 as well as the most representative pancreatic CSC markers CD133, CD24a, EpCAM and CD44.nalysis for the differentially expressed genes (DEG) of single tumours were applied based on the following comparisons: i) 1st CSCcm (CSCcm8_1), 2nd CSCcm (CSCcm8_2) and 3rd CSCcm (CSCcm8_3) to CSCcm (CSCcm8), ii) 1st CSCcm to 2nd CSCcm and iii) 2nd CSCcm versus 3rd CSCcm. Venn diagrams indicated 1st CSCcm to bear a particular transcriptome programme among the 4 groups (Figure 15).

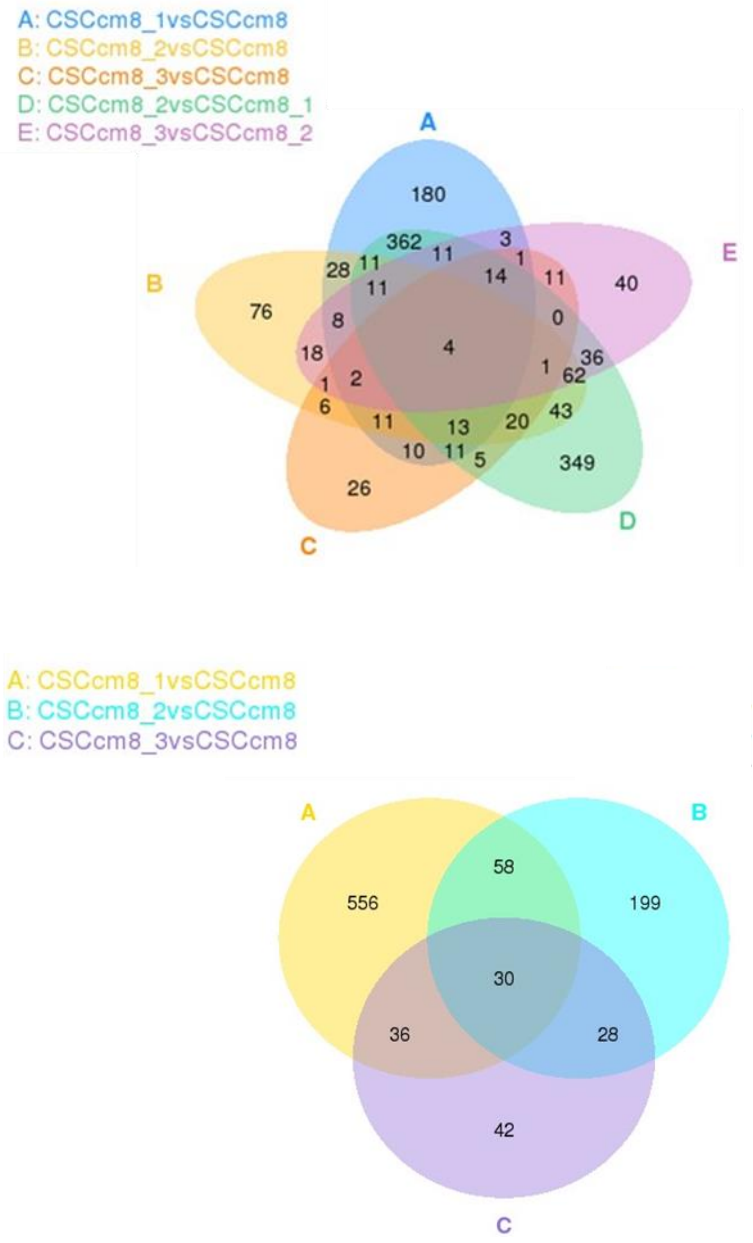


Figure 15. Molecular characterization. A) Venn diagram shows the differentially expressed genes (DEG) of the 5 possible combinations among the CSCcm PK8 lines (left), and the combination of 1st CSCcm, 2nd CSCcm and 3rd CSCcm versus the CSCcm (right).

Thus, to gain insights of the similar expression patterns DEG data were arranged in a cluster analysis using the log10(FPKM+1) value which revealed CSCcm and 3rd CSCcm to be closer in expression whereas the 2nd CSCcm slightly differed and finally corroborated 1st CSCcm

as the most differentially expressed group (Figure 16). The gene identification and their corresponding localization within the heat map suggested possible new roles for already described genes in PDAC. PDGFRb and CDKN2a which are known to participate in the development and progression of PDAC (17, 18) co-localise with the CSC markers EpCAM and CD44 which leads to question their level of implication in the acquisition of the CSC-like genotype pattern (Figure 16).

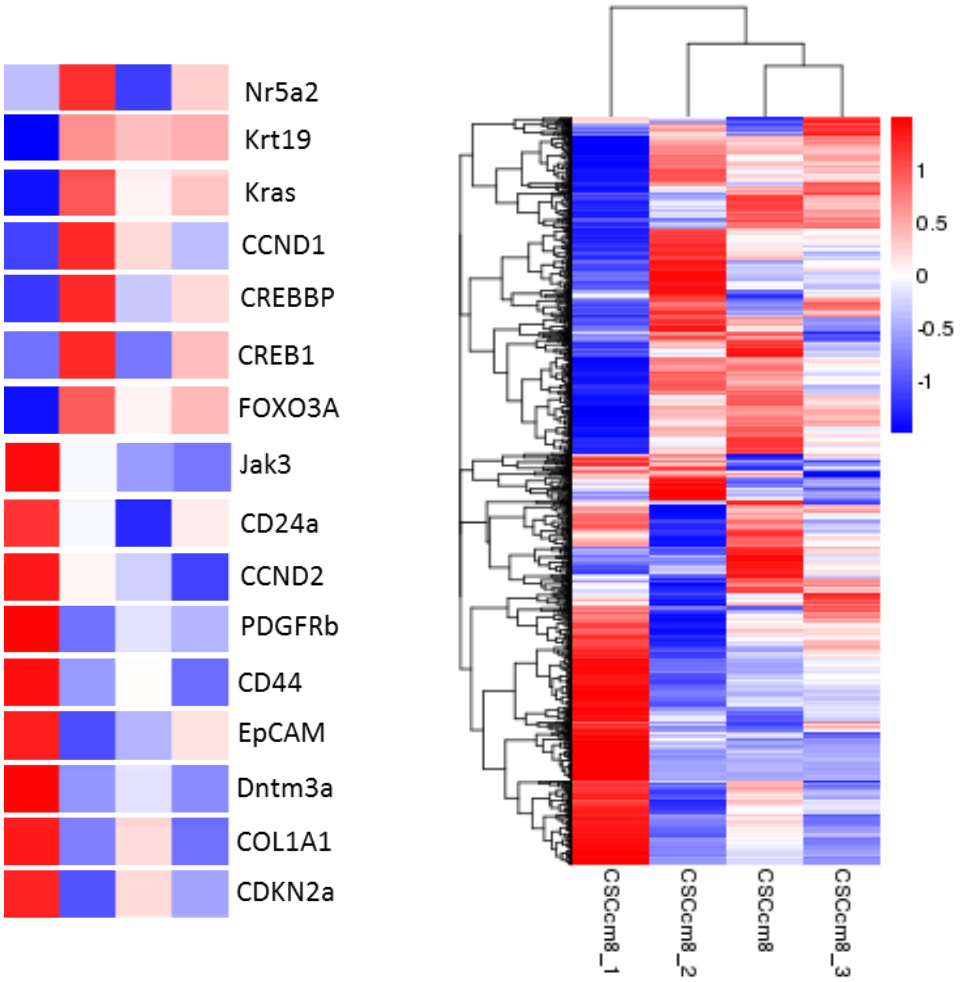
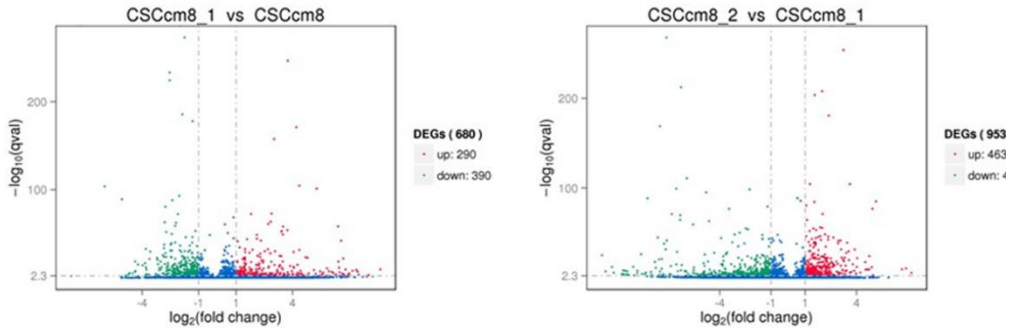
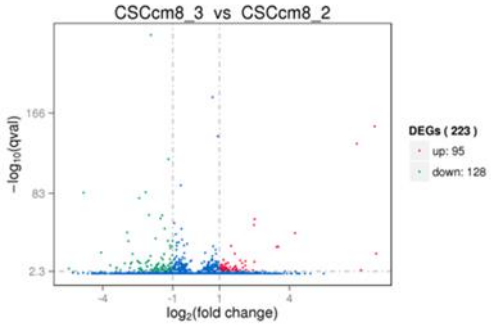
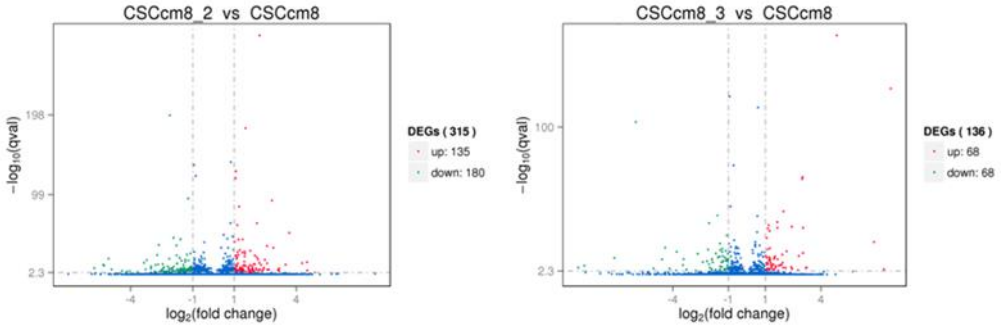


Figure 16. Representative cluster analysis among the four CSCcm PK8 lines (right). The colour range represents the log10(FPKM+1) value from large (red) to small (blue). Main oncogenes and CSCs markers implicated in PDAC carcinogenesis were enlarged (left).

In order to elucidate potential candidates involved in the malignant transformation the distribution of differentially expressed genes (DESeq) was analysed for each of the aforementioned comparisons and represented in volcano plots |(log_2 (FoldChange)|> 1&qvalue< 0.005) (Figure 17). The transcript levels of pancreatic CSC markers CD24a, EpCAM and CD44 were remarkably enhanced in 1st CSCcm whilst decreased in the subsequent generations. Instead, the popular hallmarks of PDAC KRAS, Krt19 and Myc were notably activated in 2nd CSCcm and persisted in 3rd CSCcm. Suggesting that the fate of the 1st CSCcm lineage may probably be established. The activation of Nr5a2 which its heterozygosity has been recently proposed to likely contribute to the occurrence of the PDAC (38), was sustained in 2nd CSCcm whereas in 1st CSCcm was dysregulated. Interestingly, the expression of GATA6 which have been found to be spontaneously lost in mouse model of Kras(G12V)-driven PDAC was not found in any of the groups (39). On the other hand, the original converted cell line CSCcm only differed from 2nd CSCcm in the increased expression of Krt19 and the activation of PI3K in 3rd CSCcm. Since tumours from all CSCcm lines were rich in desmoplastic stroma we sought for corresponding PSCs markers. Col8a1, Col1a1 and Col1a2 including TIMPs and Mmp2 which have been linked to tumour progression and invasion (19-21) were observed in 1st CSCcm. In addition, family members from CXC/CC chemokines Ccl2, Cxcl1 and Cxcl5 that are reported to favour the occurrence, maintenance and progression of the tumour (22) were also expressed in a correlated manner with PSCs markers. In overall, DESeq analysis consistently supported the gain of CSCs features and the established PDAC molecular pattern in the subsequent generations of CSCcm. Nonetheless a full transcriptome comparison between other RNA-seq datasets from other tumour types would be required.


CSCcm8_1 vs CSCcm8
DEGs (680)
up: 290
down: 390
CSCcm8_2 vs CSCcm8_1
DEGs (953
up: 463
down:
log2(fold change)
−log10(qval)


CSCcm8_3 vs CSCcm8_2
DEGs (223)
up: 95
down: 128
log2(fold change)
−log10(qval)


CSCcm8_2 vs CSCcm8
DEGs (315)
up: 135
down: 180
CSCcm8_3 vs CSCcm8
DEGs (136)
up: 68
down: 68
log2(fold change)
−log10(qval)

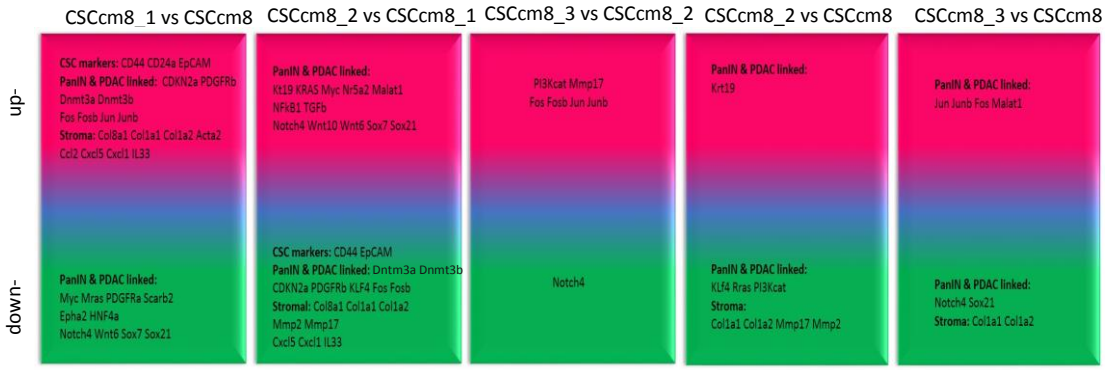

CSCcm8_1 vs CSCcm8
CSCcm8_2 vs CSCcm8_1
CSCcm8_3 vs CSCcm8_2
CSCcm8_2 vs CSCcm8
CSCcm8_3 vs CSCcm8
up-
down-
CSC markers: CD44 CD24a EpCAM
PanIN & PDAC linked: CDKN2a PDGFRb Dnmt3a Dnmt3b
Fos Fosb Jun Junb
Stroma: Col8a1 Col1a1 Col1a2 Acta2
Ccl2 Cxcl5 Cxcl1 IL33
PanIN & PDAC linked:
Myc Mras PDGFRa Scarb2
Epha2 HNF4a
Notch4 Wnt6 Sox7 Sox21
PanIN & PDAC linked:
Krt19 KRAS Myc Nr5a2 Malat1
NFkB1 TGFb
Notch4 Wnt10 Wnt6 Sox7 Sox21
CSC markers: CD44 EpCAM
PanIN & PDAC linked: Dntm3a Dnmt3b
CDKN2a PDGFRb KLF4 Fos Fosb
Stromal: Col8a1 Col1a1 Col1a2
Mmp2 Mmp17
Cxcl5 Cxcl1 IL33
PI3Kcat Mmp17
Fos Fosb Jun Junb
Notch4
PanIN & PDAC linked:
Krt19
PanIN & PDAC linked:
KLF4 Rras PI3Kcat
Stroma:
Col1a1 Col1a2 Mmp17 Mmp2
PanIN & PDAC linked:
Jun Junb Fos Malat1
PanIN & PDAC linked:
Notch4 Sox21
Stroma: Col1a1 Col1a2

Figure 17. Volcano plots for the differentially expressed genes screening (DESeq) distributed in significantly up-regulated genes (red), donw-regulated (green) and not differentially expressed (blue). Threshold set as: |log2(FoldChange)|>1 and qvalue < 0.005. Representative scheme of the up- and down-regulated genes linked to PDAC occurrence and progression.

"Mutation or not mutation-that is the question"

There is a wide range of genetic abnormalities in PDAC most of them derived from somatic single nucleotide variants (SNVs). In particular, the activation of the point mutation in Kras codon 12 has an 85-90% of prevalence in PDAC cases. Several mouse models have demonstrated that endogenous expression of oncogenic KrasG12D induces phenotypic changes at molecular and cellular levels that eventually recapitulate PDAC (7, 23-25).

The variants obtained from the screening of repeated reads showed no evidences of single point mutation in Kras codon 12 nor 13 for any of the groups. In fact, the resulting variants were found to be located in the 3'-UTR region of the oncogene and were present in all CSCcm lines. To discern whether the Kras variants had any biological relevance in the malignant transformation of the CSCcm, and due to iPSCs have a high SNPs variability, we decided to screen the mouse strains sources from where the original fibroblast was obtained previously to the iPSCs reprogramming (26). Through the database platforms http://www.informatics.jax.org and http://www.sanger.ac.uk, DBA mouse strains which are supposed to compose the 50% of the fibroblast genome were found to harbour the exact Kras SNPs as CSCcm lines. Likewise, the presence of INDELs were not localised within the oncogene (Table 2). Thereby, there were no evidences of correlation between the SNPs found in Kras and the malignant transformation.

Table 2. Chromosomal SNPs coordinates within KRAS gene for CSCcm, 1st CSCcm, 2nd CSCcm and 3rd CSCcm PK8 lines.

KRAS location: Chromosome 6: 145216699-145250239

CRHOM	POS	ID	REF	ALT
chr6	145216167	.	T	G
chr6	145217363	.	T	C
chr6	145218799	.	C	T
chr6	145218800	.	C	T
chr6	145219190	.	C	T
chr6	145219338	.	G	A
chr6	145220093	.	C	G
chr6	145220160	.	C	T
chr6	145220208	.	G	T

CHROM: Chromosome ID of SNPs

POS: Position of SNPs on corresponding chromosome

ID: The ID of this variation in dbSNP

REF: Reference genotype.

ALT: SNP genotype

Discussion

PDAC also known as pancreatic ductal adenocarcinoma is the most representative form of pancreatic cancer. PDAC is characterised by heterogeneous population of tumour cells structured in CSCs, differentiated cancer cells, tumour-associated PSCs and immune cells (1,4,6). Similarly to iPSCs, CSCs are considered as cells bearing stem cell properties that give rise to a diverse lineage of cancer cells. Driven by this convergence between CSCs and iPSCs, we opened a new avenue to generate new cell lines endowed of CSCs properties and enable to recapitulate the PDAC tumour phenotype.

Based on the observations that the xenograft tumours generated from CSCcm lines recapitulate the ductal adenocarcinoma phenotype, and that puromycin-enriched primary cultures ensured to start over from the top of the hierarchy where CSCs are residing, we can postulate that CSCcm indeed give rise heterogeneous progenies composed by more differentiated cancer cells and PSCs in a hierarchical manner. Nevertheless, it is important to note that our results have pointed out 1st CSCcm lines as the potential candidates to represent the pancreatic CSCs. Thus, even though the conditioned medium from cancer cell lines provides an appropriate microenvironment able to initiate a malignant transformation, it is not until CSCcm get in touch with an *in vivo* system that the CSCs features become robust at the level of transcriptome. Supporting this, clear experimental evidences were seen when enriched primary cultures from 1st CSCcm generated a particular cell distribution wherein specific myofibroblast-like cells arose favouring the maintenance and stability of the CSCcm colonies. Therefore, the intervention of the organisms is still essential. Certainly, this reliance on *in vivo* systems is an unresolved matter that must eventually be overcome. Recent studies in the tumour microenvironment (TME) have provided new insights about how the cancerous niche plays a significant role in disease progression sustaining cell proliferation, activating invasion and metastasis (27). It has been appreciated for some time the influence of the TME, but the precise function of each constituent remains unknown. We expect that future analysis on the components of the conditioned medium

from cancer cell lines will help to elucidate new mechanisms to optimize the conversion of CSCcm and end up being independent of the murine organisms.

Important early steps in pancreatic tumour initiation and progression are the genome reprogramming and dedifferentiation. Thus, the acino-ductal metaplasia has a relevant implication in the development of the pancreatic cancer. We observed that CSCcm tumours also generated ADM. In order to confirm the acinar phenotype the expression protein Ptf1a was sought in tumour samples and strong expression was found in the cell cluster structures. The expression of Hes1 together with Ptf1a are attributed to the characterization of centroacinar cells (CACs) which have been ascribed to bear stem-like features (7, 28). Since the RNA-seq expression quantification analysis indicate Hes1 as one of the most highly expressed genes, prompted us to question whether Ptf1a positive cells found in CSCcm tumours are either acinar or CACs. Further experiments will be required in order to clarify the real identity of the cells responsible for the ADM transition.

PDGFRb and CDKN2a were found to be highly expressed in 1st CSCcm and strongly decreased in the rest of the groups. The overexpression of PDGFRb has been linked to a poor disease-free survival promoting metastasis in a cell-autonomous manner in mutant p53 mice and glioma stem cells have been reported to preferentially express PDGFRb which its activation promotes self-renewal and its depletion completely abrogated their tumorigenicity (17, 29). Intriguingly, CDKN2a acts as a tumour suppressor and is usually aberrant in 95% of pancreatic cancer. This alteration may be due to either an abnormal methylation or several reported germline mutations which leads to its inactivation (18, 35). When SNPs in CDKN2a were screened in CSCcm lines there were no evidences of single point mutations, but a single INDEL C<CAA located in the 3'-UTR region in CDKN2a was present only in 1st CSCcm group (Supplementary Table II). Nevertheless the same INDEL was found in the murine genome but neither relevant clinical nor functional information has been described so far. Thus, the sudden enhanced expression of PDGFRb may be related to the CSCs phenotype of 1st CSCcm. On the other hand, from the results obtained for CDKN2a we could not discern properly its implication, although its

sudden downregulation in 2nd CSCcm and maintained in 3rd CSCcm may be linked to the establishment of the PDAC phenotype.

As it has been previously aforementioned, the frequency of single point mutations in Kras occur at 85-90% in pancreatic cancer (25, 30, 31). The activation of the oncogenic Kras together with its downstream effector Myc have been implicated in self-renewal and tumour plasticity events such as dedifferentiation (24). In our study we demonstrated that the tumorigenicity of CSCcm and consequent recapitulation of PDAC phenotype is not given by variants in the oncogene Kras since no formal evidences were found in SNPs analysis. However, this premature idea should be developed in extended eQTLs analysis which will provide information at the level of genotype-gene relation in addition to karyotype and genomic profiling analysis that would enable clear discrimination. MYC is generally overexpressed in PDAC. In accordance to this DESeq analysis showed increased expression of Myc in the 2nd CSCcm generation, however it is important to note that it was remarkably downregulated in 1st CSCcm. Recent findings on genome-wide DNA methylation profiling in pancreatic CSCs determine CpG sites annotated to Myc to be more methylated (5). This may be correlated with the overexpression of Dnmt3a and Dntm3b observed in 1st CSCcm and the subsequent downregulation in 2nd CSCcm. The predominant modification of DNMTs is the catalysis of the DNA methylation at 5-position cytosine. Dntm3a and Dnmt3b function as *de novo* methyltransferases and are highly expressed in embryonic cells and down-regulated in adult tissues. They have also been involved in self-renewal and maintenance of colon cancer stem cells, and pancreatic cancer patients with higher levels have been significantly attributed to have an overall lower survival (41). Thereby, the activation of Kras and Myc are required but not sufficient to originate a PDAC tumour and their turnover between 1st CSCcm and more differentiated 2nd CSCcm may be tightly related to epigenetic alterations.

In the context of inflammation, the combination of Kras and the activation of NFkB have been reported to induce the conversion of non-stem into stem cell properties of intestinal epithelial cells (IEC) through the stabilization of β-catenin (32). Despite there are not strong evidences in

pancreatic cancer models that implicate NFkB in PDAC progression (25), it was noteworthy that in 2nd CSCcm the levels of NFkB arose as Kras and Myc did. Experimental evidences are required to evaluate whether the acquisition of stem-like features may arise in a stochastic way since our model is based on iPSCs, however this fact may involve NFkB pathway in the prevalence of the CSCcm in pancreatic cancer cells progeny. On the other hand, inflammatory cells constitute an important part of the stromal tissue in pancreatic cancer and the existence of a consistent feedback between PSCs and CXC/CC chemokines family members makes to consider them as potential candidates to be effectors in the occurrence and progression of the tumour (19-22). Hence, these facts were corroborated with our DESeq data which indeed showed that the PSCs markers Col81a, Col1a1, Col1a2 were correlated with the expression of Ccl2, Cxcl1 and Cxcl5 chemokines interestingly with the stimulation of the CSCs markers overexpression. In addition, IL-33 is known to activate mast cells and stimulate pro-inflammatory cytokine production and has been found to be expressed in the nuclei of activated PSCs (36, 37). Intrapancreatic mast cells also express the cytokine stem cell factor (SCF) which plays a constitutively important role in proliferation and survival of pluripotent progenitor cells together with its receptor proto-oncogene tyrosine-kinase KIT (cKIT) (33, 34). Although pancreatic cancer cells express cKIT, its role in CSCs still needs to be elucidated. Interestingly, in the expression quantification analysis it appears to be overexpressed and DESeq results highlighted a differential expression of cKIT in 1st CSCcm indicating that it could be a potential target to abrogate the acquisition of CSCs properties. Thus, the aforementioned results notably remark the involvement of the inflammatory system as the main effector for the acquisition of the CSCs properties.

Pancreatic CSCcm is a feasible model for pancreatic CSCs which recapitulates PDAC phenotype. It is important to note that the conversion process has not been achieved under any genetic manipulation and the present study has demonstrated a preliminary analysis where the expected single point mutations were not found. Therefore, our model may provide new insights about the actual occurrence of the pancreatic cancer leading to develop different approaches for

the early detection and find new effectors in order to target CSCs and abrogate the progression of this fatidic disease.

Material & Methods

Cell culture

Human pancreatic carcinoma cell lines PK-8 and KLM-1 (RIKEN cell Bank, Japan) were cultured in RPMI 1640 Sigma containing 10% FBS and 100 U/mL Penicillin. Undifferentiated mouse iPSC lines (RIKEN Cell Bank, Japan) were maintained on feeder layers of mouse embryonic fibroblasts (MEF) mitomycin C treated (ReproCell, Japan) in iPSCs medium (DMEM D5796 Sigma, 15% FBS, 2mM L-Glutamine, 0,1mM NEAA, 50 U/mL Penicillin and 50 U/mL Streptomycin, 0,1mM 2-mercaptoethanol) supplemented with 1000 U/mL LIF, placed at 37ºC incubator, 5% CO_2 and 100% humidity. The conditioned medium (CM) was prepared following the previously protocols published by our group, Chen et al. 2012 (9), the medium was collected from confluent dishes of PK-8 and KLM-1 cell lines populations centrifuged at 1000rpm for 5min and filtered using 0,22µm diameter pore filter (Millipore, Ireland).

The conversion of the iPSCs into pancreatic cancer stem cells by CM (CSCcm) were generated as described below. For initiation of the PK-8 CSCcm and KLM-1 CSCcm lines established undifferentiated feeder-less iPSCs were seeded at $5x10^5$ cells/mL and maintained with iPSCs medium, without LIF, combined with the CM obtained from PK-8 and KLM-1 cell lines. The medium was changed every 24h and cells were sub-cultured every 3 days.

For 1st CSCcm, 2nd CSCcm and 3rd CSCcm primary cultures of PK8CM and KLM-1CM mouse allografts were cut into small pieces in 2mL HBSS and transferred into a 15mL tube containing 2mL of dissociation buffer (0,25% trypsin, collagenase IV, KSR, $CaCl_2$) and were incubated at 37ºC for 40min. The cellular suspension was transferred into a new 15mL tube and centrifuged at 1000rpm for 10min, iPSCs medium was added to terminate the digestion and again centrifuged at 1000rpm for 5min. The cell pellet was placed into appropriate volume of iPSCs medium without LIF and cells were seeded into a dish at a density of $5x10^5$ cells/mL. After 24h cultures were enriched with 1 mg/mL puromycin and medium was replaced by the combination of CM

plus iPSCs medium. Medium was changed every 24h and cells were passaged every 3 days and cells were maintained no longer than the 5^{th} passage.

Sphere formation assay

4×10^4 cells (1×104 cells/ml) were seeded on 6cm ultra-low attachment dishes (Corning incorporated, NY) with the iPSCs medium without FBS, supplied with Insulin-Transferrin-Selenium-X (ITS-X, Life Technologies, CA). After 3 days primary spheres were dissociated at single cell with dissociation buffer (0,25% trypsin, collagenase IV, KSR, $CaCl_2$) and $1x10^4$ were transferred into a new ultra-low attachment 6cm dish for 3 days more. Spheroids with diameters above 100µm were judged as self-renewing spheroids.

Animal experiments

Nude mice (Balb/c -nu/nu, female, 4- to 6- week-old) were purchased from Charlesriver, Japan. For subcutaneous transplantation 10^4, 10^5 and 10^6 cells were suspended in 100µl of HBSS buffer. The tumour size and weight were recorded every 4 days. Pancreatic orthotopic transplantation was performed as previously described by Bruns *et al*. 1999 (40) with the only variation in the number of implanted cells (10^5/20µl and 10^6/20µl HBSS). The mice were housed under specific pathogen-free conditions in accordance with the plan of animal experiments reviewed and approved by the ethics committee for animal experiments of Okayama University under the IDs OKU-2008211, OKU-2009144, OKU-2010179 and OKU-2011-305.

Histologic analysis

Tumours were fixed in 4% PFA (WAKO, Japan) for 24h at 4ºC, washed in 20% sucrose solution, embedded in paraffin-wax and sectioned for histologic examination at 5µm. Sections were stained with hematoxylin and eosin (Hematoxylin solution, Sigma-Aldrich, MO; 0.5% Eosin Y, Sigma Aldrich, MO).

Immunohistochemistry (IHC)

Before staining, paraffin was removed from sections using an oven at 60ºC for 30min, rehydrated and underwent antigen retrieval by treatment of 10mM citrate buffer (pH6.5) in a water bath at 100ºC for 30min. After hydrogen peroxide blocking (3% H_2O_2), slides were blocked with PBS + 1.5% normal serum, incubated with primary antibodies overnight at 4ºC, rinsed with PBS, incubated with secondary antibody. Detection was accomplished using Ellite anti-rabbit and anti-mouse ABC staining Vectastain kit (Vector, MI) and 3,30-diaminobenzidine tetrahydrochloride (DAB, Vector, MI). Counterstain was achieved with hematoxylin. Incubation of primary antibodies was carried out in 2.5% normal serum for rabbit polyclonal CD133 (1:100, #NB120-16518, Novus Biologicals, USA), rabbit monoclonal GFP (1:200, #2956, Cell Signaling, MA), rabbit monoclonal MUC1 (1:100, #ab15481, Abcam, UK), mouse monoclonal MUC5aC (1:200, #NCL-MUC-5AC, Novocastra, Leica Biosystems Newcastle, UK), PTF1a mouse monoclonal (1:100 ,#sc-393011, Santa Cruz Biotechnology INC., Europe). Slides served as negative controls were incubated with PBS buffer.

RNA preparation and RT-qPCR

Total RNA was isolated by RNeasy Mini Kit (QIAGEN, Germany) and treated with DNase Amplification Grade (Invitrogen, CA). cDNA synthesis was performed using SuperScript III First strand kit (Invitrogen, CA). RT-qPCR was performed with Cycler 480 SYBR Green I Master mix (Roche, Switzerland). Primers used are listed below:

Name	Primer sequence (5‘ > 3‘)
Fw CD24a	TTCTGGCACTGCTCCTACC
Rv CD24a	GCGTTACTTGGATTTGGGGAA
Fw CD133	CCTTGTGGTTCTTACGTTTGTTG
Rv CD133	CGTTGACGACATTCTCAAGCTG
Fw B-Catenin	TCCCATCCACGCAGTTTGAC

Rv B-Catenin	TCCTCATCGTTTAGCAGTTTTGT
Fw PI3K	TGGGACCTTTTTGGTACGAGA
Rv PI3K	AGCTAAAGACTCATTCCGGTAGT
Fw Akt	GGCCCCTGACCAGACCTTA
Rv Akt	GATAGCCCGCATCCACTCTTC
Fw Pdx1	ATTCTTGAGGGCACGAGAGC
Rv Pdx1	GGTCCGTATTGGAACGCTCA
Fw Foxa2	TGGTCACTGGGGACAAGGGAA
Rv Foxa2	GCAACAACAGCAATAGAGAAC
Fw Hes1	CGGCATTCCAAGCTAGAGAAGG
Rv Hes1	GGTAGGTCATGGCGTTGATCTG
Fw Kras	CAAGAGCGCCTTGACGATACA
Rv Kras	CCAAGAGACAGGTTTCTCCATC
Fw EpCAM	CTGGCGTCTAAATGCTTGGC
Rv EpCAM	CCTTGTCGGTTCTTCGGACTC
Fw Tg Klf4	GCGAACTCACACAGGCGAGAAACC
Rv Tg Klf4	TTATCGTCGACCACTGTGCTGCTG
Fw Tg c-Myc	CAGAGGAGGAACGAGCTGAAGCGC
Rv Tg c-Myc	TTATCGTCGACCACTGTGCTGCTG

RNA-seq library construction and sequencing

Isolation of total RNA was performed using QIAGEN RNeasy kit. RNA samples were prepared for sequencing using Illumina TruSeq RNA Sample Preparation Kit and were sequenced in an Illumina HiSeq 2500. Sample preparation, RNA-sequencing and Bioinformatic analysis were carried out by Fligen, INC. (Novogene, Nagoya Japan).

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