EVALUATION OF CANCER-STEM CELLS IN DIFFERENT MODELS OF PANCREATIC DUCTAL ADENOCARCINOMA

S.S.D. MED/08 – MED/18

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ABSTRACT
Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease, mainly due to late diagnosis and its intrinsic resistance to available treatments. Similarly to many solid cancers, PDAC contains a rare population of highly tumorigenic ‘stem-like’ cells (CSC, cancer-stem cells), which have been shown to possess distinct features as compared to more differentiated cells composing the bulk of a tumour. While more differentiated cells are thought to succumb to the effects of chemotherapy, CSCs survive drug treatments and cause relapses by rapidly repopulating tumours. However, CSCs represent only a small fraction (1-5%) of neoplastic cells in tumour, which makes their study challenging. Previous studies have shown that pancreatic CSCs can be enriched in vitro as anchorage-independent spherical colonies expressing stem cell markers (e.g., CD133 and autofluorescence). In vitro three-dimensional (3D) cultures, including organoids, are emerging as novel systems to study tissue development and organogenesis. Here, we report the characterization of CSCs in pancreatic tumour cultures established from patient derived xenograft (PDX) of PDAC. We established organoid cultures from four PDX-tumours and showed that they are epithelial cultures enriched for cells expressing stem cell markers (e.g., autofluorescence) and displaying high expression of pluripotency-associated genes as compared to their corresponding more differentiated monolayer cell cultures. Most importantly, following transplantation in immunodeficient mice, organoids were capable of recapitulating the morphological heterogeneity of the parental tumour. Our results highlight the enhanced stemness potential of PDAC organoids and their potential value as an in vitro model system to study CSCs. 3D systems have recently emerged as advanced drug screening platforms as, unlike the 2D cell cultures, organoids more adequately mimic the cell and tissue architecture observed in vivo. Our preliminary data show that PDAC organoids are more resistant than conventional monolayer cell cultures to standard chemotherapy with gemcitabine and abraxane aligning them with the resistance/sensitivity profile usually observed in vivo. Thus, pancreatic organoids can be used to model PDAC and as drug screening platforms to predict clinical responses and personalised cancer treatments.
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABCG2</td>
<td>ATP-binding cassette sub-family G member 2</td>
</tr>
<tr>
<td>ABC Transporter</td>
<td>ATP-binding cassette Transporter</td>
</tr>
<tr>
<td>ABX</td>
<td>Abraxane</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>ALDH-1</td>
<td>Aldehyde dehydrogenases family 1</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumine</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
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<tr>
<td>CSC</td>
<td>Cancer Stem Cell</td>
</tr>
<tr>
<td>CSCs</td>
<td>Cancer Stem Cells</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Chemokine receptor type 4</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6- diamidino-2-phenylindeole</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Dulbecco’s Modified Eagle-Medium: Nutrient Mixture F-12</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>epithelial growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epithelial growth factor receptor</td>
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<tr>
<td>EpCAM</td>
<td>Epithelial cell adhesion marker</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovin serum</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-Fixed, Paraffin-Embedded</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>LRP</td>
<td>Lipoprotein receptor related protein</td>
</tr>
<tr>
<td>GEM</td>
<td>Gemcitabine</td>
</tr>
<tr>
<td>GEMMS</td>
<td>Genetically engineered mouse models</td>
</tr>
<tr>
<td>LGR5</td>
<td>Leucine-rich repeat-containing G-protein-coupled receptor 5</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>OCT 3/4</td>
<td>Octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>PanIN</td>
<td>Pancreatic intraepithelial neoplasia</td>
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<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDX</td>
<td>Patient derived xenograft</td>
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<tr>
<td>RT-qPCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SMAD4</td>
<td>SMAD family member 4</td>
</tr>
<tr>
<td>SOX2</td>
<td>SRY (sex determining region Y)-box 2</td>
</tr>
<tr>
<td>STREP</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>TCIs</td>
<td>Tumour-initiating cells</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
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</table>
INTRODUCTION
1 Pancreatic cancer

According to the National Institute of Cancer, pancreatic cancer is a malignant growth of the different cell types that form the pancreas ([https://www.cancer.gov/types/pancreatic](https://www.cancer.gov/types/pancreatic)). Pancreatic cancers can be divided into exocrine and endocrine tumours, depending on the cell origin of the tumour. Exocrine pancreatic tumors are further divided into adenocarcinomas and a variety of other pancreatic neoplasms (Bosman et al. 2010). This work is focused on pancreatic ductal adenocarcinoma, the most frequent and lethal type of pancreatic cancer.

1.1 Pancreatic Adenocarcinoma

The most common form of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC), which accounts for approximately 90% of all pancreatic cancer cases (Morris et al., 2010). PDACs usually arise from precursor lesions in the pancreatic ducts, which present with mucinous production and generally form cysts (mucinous cystic neoplasms and intraductal papillary mucinous neoplasms) ([Figure 1](#)). Mucinous cystic neoplasms form in the pancreatic tail and rarely become invasive, whereas intraductal papillary mucinous neoplasms are localized in the pancreatic head and can progress to invasive adenocarcinoma and metastasize (Morris et al., 2010). The lesions that most frequently lead to PDAC are the pancreatic intraepithelial neoplasias (PanINs). PanINs are flat epithelial lesions confined to pancreatic ducts ([Figure 2](#)). They have been extensively characterized, and the histological changes observed in the small pancreatic ducts correlate with genetic alterations primarily involving activation of the KRAS oncogenic pathway. KRAS activation is followed by a sequence of mutations that will eventually lead to invasive PDAC, including inactivation of several key tumor suppressors such as CDKN2A and P53 (Bardeesy and DePinho, 2002; Morris et al., 2010). Non-ductal neoplasms account for approximately 10% of pancreatic cancer cases and are divided in pancreaticoblastoma, acinar cell carcinoma and solid-pseudo papillary neoplasms.
These tumours are both histologically and genetically distinct from adenocarcinomas since they develop following mutations in the β-catenin pathway, highlighting the importance of particular molecular pathways to different types of pancreatic tumours (Table 1). Patients suffering from non-ductal neoplasms have better prognosis, with significantly longer survival than patients with adenocarcinomas (Morris et al., 2010).
<table>
<thead>
<tr>
<th>Types</th>
<th>Cell of origin</th>
<th>Mean survival</th>
<th>Description</th>
<th>Histological features</th>
<th>Genes involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinar Cell Carcinoma (ACC)</td>
<td>Acinar cells</td>
<td>18 months</td>
<td>Very rare; production of lipase</td>
<td>Cell clusters close to the lumen</td>
<td>APC, β-Catenin, CTNNB1, KRAS, P53, BRAF, BRCA2</td>
</tr>
<tr>
<td>Pancreatico blastoma</td>
<td>Acinar cells</td>
<td>Over 4 years</td>
<td>Primarily in children under the age of 10</td>
<td>Clusters of squamoid cells</td>
<td>APC, β-Catenin, CTNNB1</td>
</tr>
<tr>
<td>Solid Pseudo-papillary Neoplasm</td>
<td>Primordial cells</td>
<td>Over 3 years</td>
<td>Found in women in their 30’s, curable with surgery</td>
<td>Dyscohesive cell sheets, often necrosis</td>
<td>APC, β-Catenin, P53, SMAD4</td>
</tr>
<tr>
<td>Intraductal Papillary-Mucinous Neoplasm (IPMN)</td>
<td>Mucin-producing cells</td>
<td>Curable if detected early</td>
<td>Papillary, projection into the duct. Precursor for PDAC</td>
<td>Dysplasia; villi growing from large ducts</td>
<td>KRAS, P53, PIK3CA, STK11/LKB1</td>
</tr>
<tr>
<td>Mucinous Cystadenocarcinoma (MCN)</td>
<td>Mucin-producing cells; no ductal involvement</td>
<td>Long term</td>
<td>Rare malignant, precursor for PDAC</td>
<td>Cystic tumor, filled with mucin</td>
<td>KRAS, GNAS, RNF43</td>
</tr>
<tr>
<td>Pancreatic Intra epithelial neoplasias (PanIN)</td>
<td>Epithelial cells</td>
<td>Curable if detected early</td>
<td>Progressive lesions; precursor for PDAC</td>
<td>Mucin production, small ducts</td>
<td>KRAS, CDKN2A, P53, SMAD4, BRCA2</td>
</tr>
<tr>
<td>Ductal Adeno carcinoma (PDAC)</td>
<td>Mainly ductal cells, but acinar and islet cells can lead to it</td>
<td>6 months</td>
<td>90% of pancreatic cancer</td>
<td>Poorly to moderately differentiate gland structures</td>
<td>KRAS, CDKN2A, P53, SMAD4, ATM, BRCA2, PALB2, CTNNB1</td>
</tr>
</tbody>
</table>
Figure 1. Anatomy of human pancreas (Image from OpenStax, *Exocrine and Endocrine Pancreas*, 2013, http://cnx.org/content/col11496/1.6/. Licenced under CC BY 3.0)
Figure 2. Progression model for pancreatic cancer (Adapted from Klein et al., 2002).

1.2 Epidemiology

Pancreatic ductal adenocarcinoma is only the tenth most frequent form of cancer in the United States, but it is currently the fourth leading cause of cancer deaths. There was an estimated 53,070 new PDAC cases for 2016, with high predicted rates of mortality (41,780 estimated deaths for 2016) (American Cancer Society). PDAC is so lethal because it is virtually asymptomatic in its early stages; consequently it escapes diagnosis until the cancer is locally advanced or metastatic (Hustinx et al., 2005). As such, prognosis and survival are poor for patients suffering from this disease, with a 5-year survival at just 6%. The only established “curative” option for PDAC patients is resective surgery to remove the primary tumour. Unfortunately, most patients (80%) are too far advanced to justify this surgery and are placed on largely ineffective chemotherapy regimens. When surgery is performed, it is used in conjunction with irradiation and/or chemotherapy, and the 5-year survival for patients is increased up to 25 to 35% (Oettle et al., 2007). While surgery yields a significant improvement in outcome, most patients still succumb quickly to the disease because PDAC tumours are often minimally responsive or resistant to chemotherapeutic treatments (Hidalgo, 2010).
1.3 Treatment Options

The standard of care in PDAC therapy for all patients, regardless of whether or not surgery was performed, is a regimen that includes the nucleoside analogs gemcitabine (GEM) and/or 5-fluorouracil (5-FU). These drugs have been the best options for PDAC patients even though their effectiveness is limited and they are estimated to extend lifespan by only a matter of weeks (Burris, 2005). Several single agent and GEM-based combination therapies have been tested (Table 2), with minimal improvement in the outcome (Hidalgo, 2010; Ying et al., 2012). Sadly, the median survival for PDAC patients remains short at approximately 6 months (Hidalgo, 2010; Siegel et al., 2012). In 2011, a study published in the New England Journal of Medicine reported that a combination of 5-FU, leucovorin (also called folinic acid), irinotecan and oxaliplatin (FOLFIRINOX) increased survival of metastatic PDAC patients to 11.1 months, nearly doubling survival (median of 6.8 months) for patients on GEM therapy alone (Conroy et al., 2011).

Table 2. Available therapeutic options for the treatment of PDAC (Adapted from Han and Von Hoff, 2013).

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Control</th>
<th>Median Survival (months)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gemcitabine</td>
<td>5-FU</td>
<td>5.6</td>
<td>Burris et al., 1997</td>
</tr>
<tr>
<td>Gemcitabine + Erlotinib</td>
<td>GEM</td>
<td>6.24</td>
<td>Moore et al., 2007</td>
</tr>
<tr>
<td>FOLFIRINOX**</td>
<td>GEM</td>
<td>11.1</td>
<td>Conroy et al., 2011</td>
</tr>
<tr>
<td>Nab-paclotaxel + gemcitabine</td>
<td>GEM</td>
<td>8.5</td>
<td>Von Hoff et al., 2012</td>
</tr>
</tbody>
</table>

*GEM: gemcitabine  **FOLFIRINOX: Folinic acid + 5-FU + Irinotecan + Oxaliplatin
However, a recent phase III clinical trial combining gemcitabine with abraxane (a protein-bound form of paclitaxel) showed prolonged survival compared to gemcitabine alone (8.5 vs 6.7) (Han and Von Hoff, 2013).

The inability to detect PDAC early in its development and the lack of effective therapies highlight an urgent need to identify clinically relevant biomarkers of PDAC that will improve its diagnosis and treatment. Therefore, there is a drive to identify genes and pathways that predict a patient’s survival (prognostic markers) and responsiveness to chemotherapies (predictive markers). Such knowledge will likely help stratify patients for clinical trials testing new therapies or placement on an established treatment regimen predicted to be successful for that individual. This reflects a movement by cancer researchers and physicians towards personalized therapies in the clinical management of all cancers, including PDAC (Hanahan and Weinberg, 2000). This movement is based upon the recognition that 1) cancer is a heterogeneous disease, with each tumour bearing distinct molecular and genetic signatures, and 2) each patient has a unique genome that differentially influences tumour development and response to anticancer therapies.
2 Cancer stem cells

Cancer stem cells (CSCs), also termed tumor-initiating cells, are defined as a subpopulation of cancer cells with self-renewing capacity that possess high tumorigenic potential and can undergo multilineage differentiation to give rise to all types of cells present within the malignancies (Clarke et al., 2006). According to the CSC model, CSCs drive tumour growth and these cells are considered to be the underlying cause of tumour relapse and disease progression, also through their resistance to therapy and metastatic potential. Therefore, CSCs are currently extensively studied in various cancers.

Although the CSC hypothesis is very old (Virchow, 1855), the field of CSC research truly started about 20 years ago when Dick and colleagues published their pioneer studies demonstrating that only minor subpopulation of acute myeloid leukemia cells with CD34+/CD38− phenotype holds the potential to initiate leukemia in immunodeficient mice (Lapidot et al., 1994; Bonnet and Dick, 1997). Since then, a large body of evidence has been published that supports the essential role of CSCs in initiation and progression of several hematological malignancies and of a wide range of solid tumours (Beck and Blanpain, 2013).

2.1 Cancer stem cell model

Cellular heterogeneity is a common feature of a spectrum of human malignancies ranging from solid tumours to hematological malignancies and this intratumoral heterogeneity represents one of the greatest challenges in cancer therapeutics (Kleppe and Levine, 2014; O’Connor et al., 2014; Zellmer and Zhang, 2014; Brooks et al., 2015).

In general, two models are proposed to explain tumour growth and heterogeneity (Figure 3). According to the stochastic model (Figure 3a), all tumour cells are equivalent and proliferate to fuel tumour growth (Beck and Blanpain, 2013).
Conversely, the CSC model proposes that only certain cells contribute to long-term tumour growth (Figure 3b) and these CSCs generate, analogously to normal stem cells, more restricted progenitor cells with limited replicative/self-renewal capacity. However, it is believed that these progenitors may transiently extensively proliferate and thus constitute the bulk tumour (Kreso and Dick, 2014).
Figure 3. Tumour growth models. (a) In the stochastic model of tumour growth, all tumour cells are equipotent and stochastically self-renew or differentiate, leading to intratumoural heterogeneity. (b) In the CSC model, only a subset of cells with the capacity of long-term self-renewal is responsible for sustained tumour growth. These CSCs give rise to more committed progenitors with limited proliferative potential that eventually terminally differentiate. (c, d) Clonal evolution resulting from new somatic mutations may further increase intratumoural cellular heterogeneity in both stochastic and CSC model. As suggested Driessens et al. (2012) clonal evolution in the CSC model may also originate from intrinsic nature of CSCs themselves, as every CSC within a tumour is equally likely to clonally expand due to neutral competition between these cells (Illustrations created based on Beck and Blanpain, 2013).

It is widely accepted, that tumour cell subpopulations evolve through accumulation of genetic and epigenetic mutations, some of which may increase the fitness and survival of the individual clone, and thus promote its expansion in the respective tumour
microenvironment (Greaves and Maley, 2012). Importantly, clonal evolution may occur in both stochastic model (Figure 3c) and CSC model (Figure 3d) of tumourigenesis. Several studies have shown that differentiated or neoplastic non-stem cells are able to re-enter CSC state under certain circumstances. Such cell plasticity has been shown for breast carcinoma (Chaffer et al., 2011; Iliopoulos et al., 2011; Chaffer et al., 2013), colorectal carcinoma (Vermeulen et al., 2010; Schwitalla et al., 2013), and gliomas (Charles et al., 2010). These findings led to the proposal of a fluid CSC model where the cell hierarchy is more transient than previously suggested (Figure 4, (O’Connor et al., 2014)).

![Fluid CSC model](image)

**Figure 4.** Fluid CSC model. In this extended CSC model of tumour growth, both progenitor cells and differentiated cells are able to re-acquire self-renewal potential (indicated by red arrows), thus becoming CSCs. That means that although the cells are hierarchically organized with the respect to their stem-like characteristics in a certain time point, this hierarchy is more transient and may change during tumour progression (Illustration created based on O’Connor et al., 2014 and Beck and Blanpain, 2013).

However, recent lineage-tracing studies (Rycaj and Tang, 2015) provided clear evidence of CSCs across three different types of solid tumors: papilloma and squamous cell...
carcinoma (Driessens et al., 2012), glioblastoma (Chen et al., 2012), and intestinal adenoma (Schepers et al., 2012). In a study by Clevers and colleagues (Schepers et al., 2012), Lgr5+ intestinal stem cells were individually traced in a transgenic model of intestinal adenoma in vivo. This study demonstrated that a single Lgr5+ stem cell was responsible for the initiation and maintenance of the tumour suggesting the presence of stem cell activity within primary intestinal adenomas, as precursor to intestinal cancer.

2.2 Cancer stem cell markers

Identification and/or isolation of cells with CSC phenotype represent the essential step to study CSCs. For this purposes, various molecules have been evaluated for their ability to selectively mark CSC population. In the initial studies, different cell surface proteins were used to distinguish CSCs from other cancer cells. In mid-1990’s, the research group of John Dick sorted acute myeloid leukemia cells for CD34+/CD38− phenotype and showed that this cell fraction exhibited significantly higher capacity to initiate leukemia after injection into immunodeficient mice (Bonnet and Dick, 1997; Lapidot et al., 1994). Since then, a wide range of cell surface proteins has been proposed as CSC markers in many types of tumours. The first evidence for the existence of CSCs in pancreatic cancer was provided by Li et al. (2007), through the identification of a highly tumourigenic CD44+CD24+EpCAM+ subpopulation using a xenograft model of immunocompromised mice for primary human pancreatic adenocarcinoma. This subpopulation was able to generate tumors from as few as 10^2 cells in 50% of the animals, showing a high tumourigenic capacity. By the contrast, CD44−CD24−EpCAM−, the negative population for these markers, was not capable to generate tumours until 10^4 or more cells were implanted. CD44+CD24+EpCAM+ cells displayed typical stem cell phenotypes, such as self-renewal capacity, generation of progenies and recapitulation of the morphological heterogeneity of the parental tumour from which they were derived.
Using a different cell surface marker, Hermann et al. (2007) showed that the expression of CD133 in freshly isolated primary human pancreatic tumours identified a population with self-renewal capacities, and most importantly, exclusive *in vivo* tumourigenicity. Importantly, CD133+ cells maintained their tumour-initiating capability during serial passaging *in vivo*. Interestingly, they also showed that the CD44+CD24+EpCAM+ subpopulation partially overlapped with the CD133+ population. Distinct subpopulation of CD133+ cells that co-expressed CXCR4 was further identified in the invasive front of the PDAC tumors. These CD133+CXCR4+ cells were shown to have migratory capacity *in vitro* and were demonstrated to be essential for the metastatic phenotype of the PDAC *in vivo*. Although CD133+CXCR4− formed tumours at the same rate, only mice injected with CD133+CXCR4+ cells developed metastases. In accordance with these results, another study showed that CXCR4 is expressed in pancreatic intraepithelial neoplasias (PanIN) and its expression increases during PanIN progression towards invasive carcinoma (Thomas et al., 2008). The possible prognostic significance of CXCR4 in PDAC was further confirmed by a meta-analysis study showing correlation between CXCR4 expression and poor prognosis (Krieg et al., 2015). More importantly, strong association of CXCR4 expression and metastatic disease was identified in this study. Consistent with these findings, previous experimental data demonstrated increased proliferation and invasiveness of pancreatic cancer cells after induction of CXCR4 by its ligand CXCL12 (Shen et al., 2013). Although CD133 was initially suggested as a CSC marker in PDAC (Hermann et al., 2007), subsequent studies argued against the usefulness of this protein alone to specifically identify pancreatic CSCs. Immervoll et al. (2008) showed that CD133 is expressed not only in pancreatic cancer cells but also in normal pancreas. Moreover, no correlation of CD133 and patient survival was found in several studies (Immervoll et al., 2008; Kure et al., 2012). Co-expression of CD44 and CD133 was then proposed as more specific phenotype of CSCs (Immervoll et al., 2011), and was shown to predict worse survival in PDAC patients (Hou et al., 2014; Chen et al., 2012). However, significance of CD133 expression in PDAC tumorigenesis has been recently supported by two independent studies reporting CD133 as efficient negative prognostic factor (Kim et al., 2012; Li et al., 2015).
Additional markers have also been used for the characterization of CSCs: ALDH-1 (Aldheyde Dehydrogenase-1) (Feldmann et al., 2007; Jimeno et al., 2009; Rasheed et al., 2010) has been associated with a high tumourigenic population in pancreatic cancer, although more recent data suggest an abundant expression of ALDH-1 in normal pancreas tissue as well (Deng et al., 2010), which may compromise the specificity of ALDH-1 as a marker for pancreatic CSCs. Indeed, ALDH-1 can be used for tumours whose normal tissue expression of ALDH-1 is limited or restricted, such as breast, lung, ovarian or colorectal tumors, or for circulating CSCs (Table 3).

Table 3. Cancer stem cell markers for pancreatic cancer (Adapted from Dorado et al., 2011).

<table>
<thead>
<tr>
<th>Pancreatic Ductal Adenocarcinoma</th>
<th>Markers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour-initiating population</td>
<td>EpCAM+ CD44+ CD24+</td>
<td>(Li et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>CD133</td>
<td>(Hermann et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>ALDH-1</td>
<td>(Feldmann et al., 2007, Jimeno et al., 2009, Rasheed et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Side Population/ABCG2</td>
<td>(Kabashima et al., 2009)</td>
</tr>
<tr>
<td>Migrating cancer stem cells</td>
<td>CD133+CXCR4+</td>
<td>(Hermann et al., 2007)</td>
</tr>
</tbody>
</table>

Pancreatic CSCs are subject to regulation by some of key embryonic stem cell (ESC) transcription factors. ESC transcription factors are important DNA-binding proteins present in both embryonic and adult somatic cells. The critical role of these factors in reprogramming processes makes them essential not only for embryonic development but also tumorigenesis. Recently, it has been shown that these ESC markers SOX2, OCT4, and NANOG are expressed in PDAC and that these transcription factors may associate with drug resistance, metastasis and overall worse prognosis (Herreros-Villanueva et al., 2014). Regarding pancreatic CSCs, particularly SOX2 expression
seems to be crucial for stem-like features of PDAC cells (Herreros-Villanueva et al., 2013; Singh et al., 2015). Overexpression of SOX2 \textit{in vitro} induced cell dedifferentiation and promoted EMT reprogramming, which is necessary for PDAC progression. Moreover, CD44+/EpCAM+ cell fractions isolated from two different tumour samples were enriched for SOX2-positive cells (Herreros-Villanueva et al., 2013). Thus, SOX2 may serve as potential CSC marker in PDAC.

While numerous cell surface proteins have been positively evaluated in certain settings, the expression levels of many of these markers can drastically change based on environmental conditions (e.g. tumour digestion, cultivation in different conditions, xenografting), and their expression is neither exclusively nor reproducibly linked to a functional cancer stem cell phenotype (Hermann et al., 2010). Recently an intrinsic autofluorescent phenotype has been identified in CSCs and was subsequently established as a novel and functionally relevant tool to isolate and characterize pancreatic CSCs down to single cell level (Miranda-Lorenzo et al., 2014). Has been shown that this subpopulation of autofluorescent cells could be excited and emitted at 490 and 532 nm, respectively (\textbf{Figure 5}).

\textbf{Figure 5}. Spectrum of autofluorescence in primary pancreatic cancer cells (Adapted on Miranda-Lorenzo et al., 2014).
Specifically, these autofluorescent cells were markedly enriched during chemotherapy, strongly expressed pluripotency-associated genes, and were highly invasive both *in vitro* and *in vivo.*

This distinct inherent cancer stem cell property represents a novel biological feature that is traceable in real time and provides unprecedented robustness and power for the identification and purification of CSCs without the use of antibodies nor any kind of manipulation, thus drastically reducing experimental errors and artefacts. While surface marker panels are regularly tested for only certain cancer types, autofluorescence has already been shown to identify CSCs across many tumor types including pancreatic, breast, lung, liver and colorectal cancer (Miranda-Lorenzo et al., 2014).

### 2.3 Spheroid models for study cancer stem cells

Spheroid cancer models represent a major 3D *in vitro* model that has been described, over the past 4 decades, regarding cancer stem cell research. The terms “tumour spheroid” or “tumourspheres” (Gupta et al., 2009; Lin et al., 2011) were used to describe CSC spheres issued from different types of cancer having a large panel of derived names. Cultivation of CSC as a free-floating sphere (tumoursphere) was first described in brain tumours by Singh et al. (2003). Initially, the quantification and characterization of such floating spherical aggregates had been developed for normal neural stem cells grown as neurospheres, in which a single cell is able to give rise to a sphere by clonal expansion (Reynolds and Weiss, 1992; Uchida et al., 2000). In the following years, spheres were developed from a wide range of solid tumours, including breast (Ponti et al., 2005), lung (Eramo et al., 2008), colon (Ricci-Vitiani et al., 2007), prostate (Collins et al., 2005), pancreas (Li et al., 2007), and ovarian (Zhang et al., 2008) cancers, under that same assumption that “sphere assays” enable measuring self-renewal capacity. In addition to the identification of cancer stem-like properties, spheres derived from PDAC were proved to be resistant to chemotherapy and tumourigenic agents (Lonardo et al., 2015). Tumourspheres have been proven to be an excellent
model for enriching the CSC fraction but not for studying intrinsic properties of CSCs related to their 3D architecture. Furthermore, this system does not fully reproduce the tumour from which the cells are derived, especially its structure and/or microenvironment (Kim et al., 2012).

2.4 Implication for clinical practice

As proposed by CSC model, intratumoural heterogeneity is generated by CSCs which give rise to the different populations of cells. More differentiated cells, may extensively proliferate for a certain time period, and are thought to represent the tumour bulk; however, this may not be true in all cases, as suggested by Driessens et al. (2012). Driessens and colleagues, using clonal analysis in invasive squamous cell carcinoma, showed a different pattern of behaviour, consistent with geometric expansion of a single CSC population with limited potential for terminal differentiation. This study presents the first experimental evidence for the existence of CSCs during unperturbed solid tumour growth (Driessens et al., 2012).

Current conventional anti-cancer therapies target the tumour bulk, but have limited or no effect on the CSCs. If CSCs are the primary drivers of tumourigenesis and metastasis, then effective anti-cancer therapies must target these cells (Figure 6).

In clinical practice, patients often relapse and their prognosis is poor despite the initial remission of the tumour. CSCs are considered to be the cause of the observed tumour recurrence because of their resistance to therapy and their increased metastatic potential (Shamir and Ewald, 2014; Cojoc et al., 2015). Several mechanisms of this resistance were suggested: (i) efflux of chemotherapeutic mediated by upregulation of ABC transporters; (ii) high ALDH activity that allow CSCs to quickly metabolize different chemotherapeutics; (iii) enhanced response to DNA damage and prevention of this damage by efficient scavenging of reactive oxygen species; (iv) autophagy that enables CSCs to overcome microenvironmental insults like hypoxia, starvation or treatment; (v) microenvironmental stimuli provided by the specific CSC niche (Cojoc et al., 2015).
Targeting CSCs itself or exploiting these mechanisms of CSC resistance might improve cancer treatments (Chen et al., 2012). Nevertheless, to achieve these improvements, a key step is to identify and characterize the targets of such treatments – CSCs – in the respective tumour types.

**Figure 6.** Implications of CSCs in cancer treatment. Conventional anti-cancer therapies that kill primarily non-tumourigenic cells (blue, gray) can shrink the tumour, but will not eradicate the tumour because these therapies do not target CSCs, which will eventually regenerate the tumour or initiate metastases. CSC-targeted therapies represent potential treatment improvements because they will kill or differentiate CSCs, thus targeting tumourigenesis, tumour growth and tumor metastasizing. However, it is evident that treatment combining both CSC-targeted and conventional therapy will be necessary to completely eradicate the tumour (Illustration created based on Pardal et al., 2003).
3 Three-dimensional organoid cultures

3.1 A brief introduction on organoids

In vitro three-dimensional (3D) cultures are emerging as novel systems to study human tissue development and disease. The 3D culturing of normal cells was introduced in the early 1970s when James Rheinwald and Howard Green described the first long-term culture: the formation of Keratinizing colonies from single cells. They combined freshly isolated keratinocytes with irradiated mouse 3T3 fibroblasts. As in stratified skin, cell division was confined to the basal layer of the growing clones, while surficial layers consisted to terminally differentiating keratinocytes that gradually developed a cornified cell envelope. While the term “organoid” was not used in these pioneering studies, Rheinwald and Green were the first to reconstitute 3D tissue structure from cultured human stem cells.

The term “organoid” has historically been used loosely to encompass the 3D organotypic cultures derived from primary tissue, established cell lines, as well as whole or segmented organs such as organ explants consisting of multiple tissue types (Shamir and Ewald, 2014). An organoid is defined as a 3D structure grown from stem cells and consisting of organ-specific cell types that self-organizes through cell sorting and spatially restricted lineage commitment (Clevers, 2016).

In semisolid matrices, epithelial cells can develop polarized structures as a result of the assembly of cell-cell contacts and cell/matrix interactions that simulate the basement membrane. Most of the documented organoid cultures contain functional tissue units that lack the mesenchymal, stromal, immune and neural cells that intersperse the tissue in vivo. These organoids rely on artificial extracellular matrices (ECM) to facilitate their self-organization into structures that resemble native tissue architecture.

Unlike more traditional in vitro cultures, organoids are similar to primary tissue in both their composition and architecture, harbouring small population of genomically stable cells, can be expanded indefinitely, cryopreserved as biobanks and easily manipulated using techniques similar to those established for traditional 2D monolayer culture.
Organoids represent an important bridge between traditional 2D cultures and \textit{in vivo} mouse/human models, as they are more physiologically relevant than monolayer culture models and are far more amenable to manipulation, signaling pathways and genome editing than \textit{in vivo} models (Huch et al., 2013; Sato et al., 2011; Fatehullah et al., 2016). Researchers have long known of the self-organizing capacity of cells and have harnessed this ability to generate 3D cultures from primary tissues, but the development of the intestinal organoid culture system in 2009 was a major technological advance for the stem cell field. Unlike previous systems, this new method made use of our knowledge of endogenous intestinal stem cell niche components to deliver a well-defined, stable culture system capable of sustaining the long-term growth of near-physiological epithelia from purified Lgr5+ stem cells or isolated crypts. The culture system was surprisingly simple, using Matrigel as an ECM substitute, supplemented with growth factors constituting key endogenous niche signals: WNT, a Frizzled/LRP (lipoprotein receptor related protein) ligand; Noggin, a BMP (bone morphogenetic protein) inhibitor, to allow for stem cell expansion; R-spondin, an LGR4/5 (leucine-rich repeat-containing G-protein-coupled receptor 4/5) ligand, a WNT agonist to maintain stem cell populations; and EGF (epithelial growth factor), an EGFR ligand, to promote cell proliferation. Remarkably, these organoids faithfully recapitulated the \textit{in vivo} tissue architecture and contained the full complement of stem, progenitor and differentiated cell types. The system was subsequently adapted for generating human intestinal organoids, as well as organoids from other organs harbouring Lgr5+ stem cells, including the colon, stomach and liver (Sato et al., 2011; Huch et al., 2013).

\subsection*{3.2 Tumour organoids as potential model for drug screening}

As recently demonstrated, intestinal organoids have unique features as they efficiently form, self-renew, and expand long-term while remaining genetically stable (Sato et al., 2011). Using this system, organoids can be indeed readily established from surgically
resected intestinal tissue and endoscopic biopsies of patients suffering from adenomas and adenocarcinomas (Sato et al., 2011).

Patient-derived organoids represent an important resource for developing personalized treatment. Cancers have many subtypes, which display different combinations of genetic alterations. This diversity is important to understand in patients: cancer drugs specifically target different cellular pathways, so depending on the genetic background of the person and of the tumour, patients’ response to therapy may vary. Recently, Ogawa et al. (2015) were able to correct CFTR (cystic fibrosis transmembrane conductance regulator) misfolding and translocation to cell membranes in patient-derived cholangiocyte organoids, using inhibitors to reduce misfolding and stabilize the protein. This demonstrates the utility of the organoids for testing and screening novel compounds to treat various conditions. In vitro amplification of patient organoids from disease-site biopsies can deliver sufficient material for deep sequencing to reveal causal mutations, or for in-depth phenotypic profiling to facilitate more tailored treatment regimes. Organoids open up new avenues for regenerative medicine and, in combination with editing technology, for gene therapy. The many potential applications of this technology are only beginning to be explored.

3.3 Pancreatic organoids

Establishing 3D organoids from epithelial organs required tissue-specific modifications that reflect the individual requirements and lineage commitment factors for the resident stem cell populations and their progeny.

To establish pancreatic cultures, isolated pancreatic duct fragments from adult healthy mice (Figure 7A) were embedded in Matrigel containing the ‘generic’ organoid culture factors EGF, RSPO1 and Noggin (Sato et al., 2009) supplemented with FGF10 (Bhushan et al., 2001) and Nicotinamide. Under these conditions, small duct fragments formed closed structures within 24-48h that expanded into budding cyst-like organoids (Figure 7B)(Huch et al., 2013).
Establishment of the pancreatic organoids from adult pancreatic ducts. (A) Scheme representing the isolation method of the pancreatic ducts and the establishment of the pancreatic organoid culture. The pancreatic ducts were isolated from adult mouse pancreas after digestion, handpicked manually and embedded in matrigel. Twenty-four hours after, the pancreatic ducts closed and generated cystic structures. After several days in culture, the cystic structures started folding and budding. (B) Representative serial DIC images of a pancreatic organoid culture growing at the indicated time points. Magnifications: x 10 (days 0, 2, 4, 6, and 8) and x 4 (day 10 onwards). (Illustration from Huch et al., 2013).

Importantly, engraftment of these pancreatic organoids under the kidney capsule resulted in the formation of functional pancreatic tissue containing ductal, endocrine and acinar cells, providing strong evidence that pancreatic stem cell potential resides in the adult ductal compartment (Huch et al., 2013).

Similar observations were made for human pancreatic organoids (Boj et al., 2015). Pancreatic organoids derived from wild-type mice and PDAC genetically engineered mouse models (GEMMs) accurately recapitulate physiologically relevant aspects of disease progression in vitro. Following orthotopic transplantation, these organoids were capable of regenerating normal ductal architecture, unlike other 3D model systems.
OBJECTIVES
It is widely accepted that CSCs are key players in tumor initiation, progression, drug resistance and recurrence. Therefore, targeting unique features of CSCs might represent an ideal strategy for cancer eradication. Individual surface marker, such as CD133, or combination of markers such as EpCAM/CD44/CD24 have been proposed to enrich for pancreatic cancer stem cells (CSC) in fresh tumor samples. These cell subpopulations have been found to bear exclusive tumorigenicity and resistance to chemotherapy (Hermann et al., 2007). CSCs represent only a small fraction (1-5%) of neoplastic cells in tumour, which makes their study challenging. Moreover, the scarce representation of stem cells in traditional in vitro models of PDAC prompted us to investigate novel culture systems as biological platforms to study CSCs. Recently described 3D culture methods, such as organoid cultures, have been shown to recapitulate key features of in vivo cell-growth, including self-organization and differentiation. Organoid cultures represent an innovative system for culturing primary normal and diseased tissue in vitro, thereby enabling the study of human pancreas biology, as well as of pancreatic cancer development and progression. Thus, this thesis focuses on the identification and characterization of CSCs in organoid cultures established from PDAC patient-derived xenograft tumors. Based on our preliminary data concerning expression of CSC markers in primary PDAC cultures the specific objectives of this thesis were defined as follows:

1. Establishment and functional characterization of organoid cultures from different PDAC Patient-derived Xenograft Tumours.
2. Comparison of stemness content between different 3D in vitro cultures of PDAC models, namely PDAC organoids and sphere cultures.
3. Comparison of genetic Landscape of 3D-models (organoid and sphere cultures) and matched primary tumors.
4. Assessment of PDAC organoid cultures as drug screening platform for the prediction of clinical response.
MATERIALS AND METHODS
1 ANIMAL MODELS

1.1 Study Approval

Mice were housed in the animal facility of Barts Cancer Institute (QMUL) in accordance with institutional policies and federal guidelines. All animal experiments were conducted in accordance with procedures approved by the ASRU (Animals in Science Regulation Unit) at the QMUL.

1.2 Xenografts

PDAC xenografts from patient derived samples were kindly obtained from the ARC-Net Biobank (University of Verona, Italy), Department of Surgery at the Technische Universität München (Munich, Germany) and Manuel Hidalgo’s group (CNIO, Spain). Briefly, primary tumours were cut into small fragments and then implanted subcutaneously in immunocompromised mice (NU- Foxn1\textsuperscript{nu}; Charles River, Wilmington, MA, USA) with two small tumour pieces per mouse. Once tumours reached 1cm\textsuperscript{3}, tumours were resected and re-implanted in another set of female nude mice, following the protocol described in Rubio-Viqueira et al. (Rubio-Viqueira et al., 2006), and represented in Figure 8.
Figure 8. Xenograft study workflow. Tumour samples are implanted in F1 generation and then expanded in a cohort of nude mice (Adapted from Rubio-Viqueira et al. 2006).
2 CELL CULTURE

2.1 Primary human pancreatic cancer cells

Tumour tissue originating from different PDX models was minced mechanically (gentleMACS Dissociator; Miltenyi) and enzymatically digested with Dispase II (1mg/ml; MerK Millipore) supplemented with Collagenase P (4mg/ml; Sigma) for 60 min at 37°C under constant rotation. Following digestion, the cells were passed through a series of strainers (100, 70 and 40) and the resulting single cell suspension was centrifuged for 5min at 1500rpm. Red blood cells were lysed by incubation with ACK Lysis buffer (Lonza) for 5min at RT. After neutralization of the buffer with equal volume of complete medium and centrifugation, cell pellets were resuspended and cultured in complete medium: RPMI Medium 1640 (1X) supplemented with GLUTAMAX™ (GIBCO, Life Technologies), 10% FBS, and 50 units/mL pen/strep (Invitrogen).

2.2 Sphere formation assay

Spheres were generated by culturing ~2x10⁴/ml primary pancreatic cancer cells in Ultra-Low attachment flasks (Fisher Scientific) in suspension using serum-free DMEM/F12 (GIBCO, Life Technologies) supplemented with B27 (Fisher Scientific), 20 ng/mL bFGF (PeproTech), and 50 units/mL pen/strep for a total of 7 days, allowing spheres to reach a size of >75μm. For serial passaging, 7-day-old spheres were harvested using 40μm cell strainers, dissociated into single cells with Tryspin-EDTA (Sigma), and then re-grown for 7 additional days. The CASY Cell Counter (Roche Applied Sciences) was used to quantify spheres of three different fractions, 40-80μm, 80-120μm and >120μm in diameter.
2.3 Culture and propagation of organoid cultures from pancreatic PDX models

Pancreatic organoids were generated by embedding dissociated adherent cells in Matrigel, and culturing in organoid growth medium (ADMEM/F12 medium [GIBCO, Life Tecnologies] supplemented with B27 [50X, Invitrogen], Glutamax [2mM, Invitrogen], HEPES [10mM, Invitrogen], penicillin/streptomicin [1X, Invitrogen], Nicotinamide [1.25M, Sigma], N-acetyl-L-cysteine [1.25M, Sigma], Y27632 [1.6mg/ml, Sigma], SB202190 [10µM, Sigma], Human recombinant Noggin [100µg/ml, PeproTech], A83-01 [0.5ng/ml, Abcam], R-spondin [500ng/ml, PeproTech], Human recombinant EGF [50ng/ml, PeproTech], Gastrin [10nM, Sigma], FGF10 [10ng/ml, PeproTech], and FGF2 [5ng/ml, PeproTech]). Plates were pre-coated with matrigel prior to seeding the cells. The medium was changed every 3 days. After fifteen days, organoids were incubated with Cell recovery solution (Corning) for 1 hour on ice, allowing disrupt the matrigel. After incubation, organoids were washed with 1X of PBS (Sigma) and centrifuged for 5 min at 2000rpm, 4⁰C. Cell pellets were resuspended in 2 ml of TrypLE Express (GIBCO, Life Technologies) and incubated for 5-10 min at 37⁰C. An equal volume of complete medium were added as neutralizing solution and centrifuged at 1500rpm for 5 min at RT. The supernatant was discarded and the pellet was resuspended in 1 ml of fresh complete medium. The suspension was filtered through a 50 µm mesh to remove any large cell clusters and viable cells were counted on a Neubar chamber. The appropriate concentration of cell suspension per well was calculated. This volume was taken and spun for 5min at 2.0rpm and followed by dilution into the appropriate volume of matrigel.

2.4 Organoid formation assay

In order to assess the organoid formation efficiency of different primary pancreatic cancer models, cells were seeded starting from adherent conditions in regular medium into 3D organoid culture. In a 24well plate 10.000 cells were seeded per well and were allowed to grow for 2 weeks. The medium was changed every 3 days. At endpoint, on
day 14, the organoids were recovered and counted on a Neubar chamber to evaluate the number of formed organoids per ml. For the second generation, they were reseeded in a new 24-well again in 10,000 cells per well in triplicate.

2.5 Cell treatments

In order to evaluate the chemosensitivity profile of the different PDX models, primary human pancreatic cancer cells, sphere-derived cells and pancreatic organoids were treated with Gemcitabine (100 ng/mL) and Abraxane (1 μM) for 7 days. In adherent conditions, 40,000 PDAC cells were seeded in a 24 well cell culture plate in triplicate, in 1mL of complete medium. The treatment started after 24 hrs and was maintained for 7 days. At endpoint, the viability of the cells was evaluated by the Alamar Blue viability assay as described in paragraph 2.6. For the treatment of spheres cells were seeded at a density of 10,000 cells/well in 24 Ultra-Low attachment plates in suspension in complete sphere medium. Treatment started on day 4 and maintained for 7 days. At endpoint, spheres were counted using the CASY cell counter as described in paragraph 2.2. The treatment of primary pancreatic organoids was performed in a 96 well cell culture plate in triplicate. In each well, 5000 cells were seeded and were let to grow in complete organoid medium for 7 days. The drug treatment started on day 7 and continued for 7 additional days. On the last day the viability of the organoids was assessed by the Alamar Blue viability assay.

2.6 Cell viability assay

Cell viability activity was measured by Alamar Blue Assay according to the following protocol. The medium was aspirated and the cells washed once with PBS. Alamar blue (dilution 1/250 from a stock solution of 1mg/ml) in complete medium was added in
each well and the plates were incubated for 4 hrs at 37°C. Fluorescence was read immediately after incubation on a BMG plate reader (LabTech, Germany) and it was quantified using excitation and emission wavelengths of 530-560nm and 590nm, respectively. Blank control wells containing medium and Alamar Blue dye only without cells were included as negative control. Relative fluorescence was calculated by subtracting the average of the negative controls from each of the well readings. Each experiment was carried out in four replicate wells for all conditions tested, and all the experiments were done in triplicate.
3 GENOMIC ANALYSIS

3.1 DNA extraction and qualification

DNA was obtained by QIAmp AllPrep DNA/RNA mini kit (Qiagen). Two elutions in nuclease-free water were performed to obtain a final volume of 100 µl for each sample. Next-generation sequencing (NGS) suitability of extracted DNA was evaluated and was quantified using NanoDrop-ND2000 and Qubit fluorometer (Thermo Fisher) as reported in a previous study (Simbolo et al., 2013). Furthermore, a multiplex PCR was developed according to previous study (Zamò et al., 2012) to verify DNA integrity and multiplex suitability.

3.2 Mutational analysis by next-generation targeted sequencing

Five multigene panels were used to investigate mutational status of 76 genes. The custom panels target genes selected according to recent studies (Bailey et al., 2016; Biankin et al., 2012; Waddell et al., 2015) and reported in Table 4. Twenty nanograms of DNA were used for each multiplex PCR amplification. The quality of the obtained libraries was evaluated by the Agilent 2100 Bioanalyzer on-chip electrophoresis (Agilent Technologies). Emulsion PCR to construct the libraries of clonal sequences was performed with the Ion OneTouch™ OT2 System (Thermo Fisher). Sequencing was run on the Ion Proton (PI, Thermo Fisher) loaded with Ion PI Chip v2. Data analysis, including alignment to the hg19 human reference genome and variant calling, was done using the Torrent Suite Software v.5.0 (Thermo Fisher). Filtered variants were annotated using a custom pipeline based on vcflib (https://github.com/ekg/vcflib), SnpSift (Cingolani et al., 2012), the Variant Effect Predictor (VEP) software (McLaren et al., 2010) and NCBI RefSeq database. Additionally, alignments were visually verified with the Integrative Genomics Viewer (IGV) v2.3 (Robinson et al., 2011) to further confirm the presence of mutations identified by targeted sequencing.
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3.3 Copy number variation calling

CNV analysis was performed on IonReporter 5.0 software (ThermoFisher) with the CNV single sample workflow. A specific baseline was built using normal and well characterised DNA without genomic structural aberrations (10 male DNA extracted from normal tissues). The baseline was matched to the sequences of the primary PDAC tumours samples and corresponding models to obtain CNV status of the genes analysed.
4 FLOW CYTOMETRY

4.1 Flow cytometry analysis

For flow cytometry analysis, single primary pancreatic cells were stained using different combinations of antibodies (Table 5). To evaluate autofluorescence, adherent cells and spheres were incubated with riboflavin overnight or 3hrs for organoid cells as previously published (Miranda-Lorenzo et al., 2014). DAPI was used for exclusion of dead cells and isotype matched antibodies were used as negative controls. Cells were acquired with a LSRII Fortessa Instrument (BD Biosciences) and data were analysed with FlowJo 10.0 software (Tree Star).

Table 5. List of antibodies used for the analysis of primary pancreatic cells using flow cytometry.

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5 RNA ANALYSIS

5.1 RNA extraction

Total RNA from human pancreatic cancer cells was extracted using RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. Extracted RNA was quantified using the NanoDrop machine.

5.2 RT-qPCR

One microgram of total RNA was reverse-transcribed with QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR was performed with an Applied Biosystems 7500 real-time thermocycler (Applied Biosystems) using miScript SYBR® Green PCR kit (Qiagen) as per the manufacturer’s instructions. Primers used are listed in Table 6.

Table 6. RT-qPCR primers.

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<td>atctgggctggtttcttgg</td>
<td>tgcttgacattctctgagtt</td>
</tr>
</tbody>
</table>
6 IMMUNOHISTOCHEMICAL ANALYSIS

Organoids were recovered from matrigel as previously described (paragraph 2.3). Organoid pellets were washed with 1X PBS and fixed with 4% paraformaldehyde at room temperature under constant agitation for 30 min. Following fixation the organoids were centrifuged for 5 minutes at 2000rpm and the supernatant was removed. The organoid pellet was washed with 1X PBS and resuspended in approximately 100µl of 2% of agarose. Once the agarose solidified, the samples were stored in 70% EtOH prior to paraffin embedding and sectioning. For histopathological analysis, FFPE blocks were serially sectioned (3µm thick) and stained with hematoxylin and eosin (H&E) for morphological analysis. For immunofluorescence staining the paraffin slides were deparaffinized in Xylene for 10 min and rehydrated first with 100% ethanol for 10 min, in 70% ethanol for other 10 minutes and then in distilled water. Antigen retrieval was performed by boiling the slides in citric acid buffer (pH6) or Tris-EDTA buffer (pH9), in the 2100 Antigen Retriever for ~20 min and cooling for 60 min at room temperature. The slides were then washed 3 times for 5 min each in 1x PBS and tissue sections were blocked with 3% goat serum (Sigma) in 1x PBS with 0.5% BSA (Sigma) for 1 hr at room temperature (RT). The sections were incubated with the primary antibody in PBS/0.5%BSA O/N at 4°C (Table 7) and washed with 1X PBS 3 times for 5 minutes. The following day the slides were incubated with secondary antibodies in PBS/0.5%BSA (all from Fisher Scientific) for 1 hr in the dark at RT. Finally the slides were washed in 1x PBS twice for 5 minutes and incubated in PBS supplemented with 0.5% BSA and DAPI for 30min in the dark at RT. The slides were washed twice with PBS for 5 minutes and mounted with mounting medium and, were analysed using a Fluorescent confocal microscope.
Table 7. List of antibodies used for the analysis of primary pancreatic cells using immunofluorescence.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Manufacturer</th>
<th>Antigen retrieval buffer</th>
<th>Primary antibody incubation conditions</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit polyclonal to EpCAM ab71916</td>
<td>Abcam</td>
<td>Tris-EDTA</td>
<td>1:1000 O/N 4°C</td>
<td>1:500 Alexa Fluor 488 Donkey anti-Rabbit IgG (H+L)</td>
</tr>
<tr>
<td>Mouse monoclonal [RCK108] to Cytokeratin 19 ab9221</td>
<td>Abcam</td>
<td>Citric Acid</td>
<td>1:100 O/N 4°C</td>
<td>1:500 Alexa Fluor 555 Goat anti-Mouse IgG (H+L)</td>
</tr>
<tr>
<td>Rabbit monoclonal [UMB2] to CXCR4 ab124824</td>
<td>Abcam</td>
<td>Citric Acid</td>
<td>1:100 O/N 4°C</td>
<td>1:500 Alexa Fluor 488 Donkey anti-Rabbit IgG (H+L)</td>
</tr>
</tbody>
</table>
7 IN VIVO EXPERIMENTS

Pancreatic organoids were dissociated as described in paragraph 2.3, counted and resuspended in 50 µl of Matrigel™ (Corning) and kept on ice. Organoid cells (20,000 cells) were injected subcutaneously into the left and right lower flanks of 4-to 6-week old female NOD SCID and NU (Ncr)-FoxN1nu mice. Growth of transplants was monitored weekly by palpation.
RESULTS
1 Characterization of Primary ductal cell culture

Four different PDXs (1953, 2636, 140114, 215) were used in this study. Viable specimens from early passage xenografts of 4 cases were used to generate *in vitro* models, including monolayer cell cultures, spheres and organoids. Expression of stem cell markers was assessed by flow cytometry in all cases and mRNA expression of pluripotent genes was assessed in three cases. Two cases (1953, and 140114) were transplanted in immunodeficient mice and drug sensitivity was tested in an index case (1953). Immunohistochemical analysis was performed on two cases (1953, and 140114).

1.1 Analysis of pancreatic cancer stem cell markers

It has been described by Herman et al. (2007) that primary pancreatic CSCs can be enriched *in vitro* by culturing pancreatic cancer cells as anchorage-independent three-dimensional colonies, also termed spheres. Spheres are mainly composed of differentiated cells, while a small number of cells possess stem cell-like properties including the ability to form secondary spheres. In the present study, we studied pancreatic CSCs in cells isolated from 4 different human pancreatic adenocarcinoma xenografts. Cells were isolated from early passage xenografts, and cultured as low passage adherent cells or spheres. Human pancreatic cancer cells were phenotyped by flow cytometry for the expression of CSCs markers, and as previously reported (Lonardo et al., 2011), spheres resulted enriched for CD133+ cells. In contrast, cells expressing CD44 were not consistently enriched in sphere cultures, mostly likely reflecting anchorage-independent culture conditions (Figure 9 and Supplementary Fig.1).
Figure 9. Representative flow cytometry analysis for the indicated markers in primary PDAC cells cultured and sphere cultures. Plots shown are representative of the case 1953.

Recently, it has been demonstrated in freshly digested tumours from PDAC PDXs as well as in primary patient tumours the presence of a distinct autofluorescent population which was shown to be a marker of CSCs (Miranda-Lorenzo et al., 2014). Therefore, we examined the existence of this subpopulation in our PDAC PDX tumours. We observed cells with this phenotype in PDX-derived adherent cultures, a population which resulted consistently enriched for in spheres (Figure 10 and supplementary Fig. 2).
Figure 10. Flow cytometry analysis of autofluorescent content in adherent (left) and sphere cultures (right). Autofluorescent cells are excited with a 488-nm blue laser and best selected as the intersection with filters 530/40 and 580/30.

1.2. Self-renewal capacity of pancreatic CSCs

Sphere-forming assays have been widely used to retrospectively identify stem cells based on their capacity to self-renew and differentiate at the single cell level *in vitro*. Culture of primary pancreatic cells in suspension as spheres allows cells to remain undifferentiated, thus generating spheres which are rich in stem-cells (1st generation
spheres). Due to their higher content in stem cells these spheres, following enzymatic
dissociation, have the ability to give rise to secondary spheres at a higher rate
demonstrating functionally their increased stemness (Figure 11 and Supplementary
Fig. 3).

Figure 11. Representative sphere numbers and diameters (µm) for primary cell culture over one
generation (gen.). Data are representative of 1953 primary PDAC PDX-derived in vitro cultures (n=3,
each performed in triplicate). Error bars, s.d.

1.3 Pancreatic CSCs express Pluripotency-associated genes

It has been recently shown that like stem cells, CSCs up-regulate the expression of
pluripotency-associated genes (Liu et al., 2013). QPCR analysis for the expression of
pluripotency-associated genes revealed that spheres over-expressed NANOG, KLF4,
SOX2, BMI1, and OCT3/4 as compared to the adherent cell population (Figure 12), as described previously (Lonardo et al., 2011; Miranda-Lorenzo et al., 2014).

Figure 12. QPCR analysis of pluripotency-associated genes in spheres compared to adherent cultures of different PDX-tumours.
2 Human PDAC Organoids

2.1 PDAC organoids express typical markers of primary disease

Recently, continuously proliferating, normal and tumour pancreatic organoids were derived from adult murine ductal cells (Huch et al., 2013). We optimized this approach to generate organoid cultures from primary PDX-tumours. We isolated primary cell cultures from 3 different PDAC PDX-tumours to establish organoids in 3D culture. 3D in vitro culture system for the long-term expansion of human PDAC cells was generated combining Matrigel, which acts as extracellular-matrix (ECM), with medium containing growth factors as EGF, FGFs, and Rspo1. Under these culture conditions, pancreatic cells can be expanded for months in culture. A combination of factors and nutrients critical for pancreas development induced polarized 3D structures from single cells of primary PDAC cultures. Following 15 days in 3D culture conditions, epithelial cells proliferate and organize into 3D organoid structures, which are characterized by the presence of a centrally-localized, hollow lumen (Figure 13).

![Figure 13](image_url)

**Figure 13.** Left, H&E-staining of one organoid. Phase contrast microscopy photo depicting the morphology of day 15 organoids, middle (right; higher magnification image of one organoid). Data are representative of the case 1953.
To determine the contribution of different pancreatic lineages to the organoids, we evaluated the expression of pancreatic lineage markers in these cultures. Cells within organoids were demonstrated to express epithelium-associated cytokeratin 19. Furthermore, pancreatic organoids were shown to be positive for EpCAM, confirming their epithelial lineage identity (Figure 14).

**Figure 14.** Immunofluorescent staining of 15 day-old organoids for the epithelial marker EpCAM and cytokeratin 19 (KRT19). Data are representative of a case 1953.

2.2 Mutational profile analysis identified no molecular changes in genetic landscape between primary tumour tissues and their three-dimensional models

In order to confirm the genetic stability of the 3D CSC-enriched models we performed mutational profile analysis for a list of genes commonly associated with PDAC (Bailey et al., 2016; Biankin et al., 2012; Waddell et al., 2015). Multigene sequencing achieved
mean read length of 118 base pairs and a mean coverage of 2132x, with 95.8% target bases covered more than 50x. A minimum coverage of 20x was obtained in all cases. Mutations identified in 76 genes analyzed are summarized in Table 8 and illustrated in Figure 15.

<table>
<thead>
<tr>
<th>Primary tumour sample</th>
<th>Neoplastic Cellularity</th>
<th>1608</th>
<th>1953</th>
<th>2636</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50%</td>
<td>50%</td>
<td>67%</td>
</tr>
<tr>
<td>ARID1A</td>
<td></td>
<td></td>
<td></td>
<td>Asp1219GlyfsTer9</td>
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<tr>
<td>BRCA2</td>
<td>Lys3326Ter</td>
<td>Val2728Ile</td>
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<td>KRAS</td>
<td>Gly12Val</td>
<td>Gly12Asp</td>
<td>Gly12Asp</td>
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</tr>
<tr>
<td>SMAD4</td>
<td>Ile429CysfsTer7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP53</td>
<td>Thr81AsnfsTer42</td>
<td></td>
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</table>

Table 8. Detail of somatic mutations identified in primary tumour samples.

All mutations detected in primary tissue and xenograft-tumours were observed in both 3D models (organoids and spheres). In detail: KRAS mutation was identified in all samples analysed; two samples showed mutation in BRCA2 (a nonsense and a missense mutation), while frameshift mutations were observed each in one sample in TP53, SMAD4 and ARID1A genes. Of note, sample 1608 and its models showed simultaneously mutations affecting KRAS, TP53, SMAD4 and BRCA2. No additional mutations that were present in the original tumours were observed in corresponding models.
Figure 15. Genetic profile comparison between sphere, organoid cultures and their primary tumours. The cases 1608, 1953 and 2636 are arranged from left to right. Type of sample, mutation (A) and CNV (B) of altered genes are represented according to color legend below.

2.3 Copy number variation analysis in primary tumour tissues and their three-dimensional models

Copy number variation (CNV) analysis was performed on three primary PDAC tumours and their matched models (xenografts, spheres and organoids). Homozygous deletion
and loss of the heterozygosity (LOH) affected known tumour suppressor genes \textit{CDKN2A}, \textit{SMAD4} and \textit{TP53} as depicted in \textbf{Figure 15B}. CNV profile of the three-dimensional models revealed no change compared to xenograft tumours, which they derived from, except for homozygous deletion of \textit{CDK2A} observed exclusively in the spheres but not in organoid cultures from PDX 2636. More differences at CNV level were observed when comparing primary tumours and corresponding models for the cases 1953 and 2636 (\textbf{Figure 15}), which might be due to the different neoplastic cell contents of the specimens analysed rather than to the genetic drift upon model establishment.

\textbf{2.4 Transplantation of PDAC organoids morphologically resemble PDX}

To test whether the organoid cultures could generate tumours \textit{in vivo}, we subcutaneously injected 20,000 cells from two independent organoid cultures (1953, 140114) into Nude mice (3 mice per organoid culture). All injections resulted in tumour growth within 4–7 weeks. The organoid-derived tumours maintained the histoarchitectures present in the PDX- and primary tumour from which they were derived (\textbf{Figure 16}), as well as similar expression of differentiation markers, including CK19 (\textbf{Figure 17}). Thus, organoid-derived tumours conserve histological organization, differentiation status and morphologic heterogeneity observed in primary PDACs.
Figure 16. Organization of H&E-stained human primary PDAC (left), PDX-tumour (middle) and Organoid-tumour (right panel). Data are representative of one case (1953).
Figure 17. Immunofluorescence staining of epithelial markers EpCAM and cytokeratin 19 (KRT19) in PDX- and organoid-derived tumour. Data are representative of one case (1953).
3 Characterization of PDAC-Organoid cultures

3.1 PDAC organoids are enriched for cells showing stemness

Following establishment and characterization of PDAC cell cultures, we asked whether organoids were enriched for CSCs compared to corresponding adherent cell cultures, and spheroids. Flow cytometric analysis revealed no major changes in the percentage of epithelial cells, as marked by Epithelial Cell Adhesion Molecule (EPCAM). While monolayer cell cultures from PDX-tumours contained less than 4% of CD133+ cells, approximately 30% of the organoid cells expressed CD133 (Figure 18). In agreement with this finding, organoid-derived cells exhibited higher expression of stem cell markers CD44 and CXCR4 (Figure 18) as well as increased level of autofluorescence compared to adherent and sphere-derived cells (Figure 19 and supplementary Fig. 4).

![Figure 18](image-url). Flow cytometry analysis for markers EpCAM, CD133, CD44, CXCR4 in adherent, sphere and organoid cultures.
Figure 19. Flow cytometry analysis of autofluorescent content in PDAC organoid cells. Autofluorescent cells are excited with a 488-nm blue laser and best selected as the intersection with filters 530/40 and 580/30.

In order to further assess stemness of organoids, an assay similar to the sphere formation described previously was performed. In detail, starting from adherent cultures, which are considered to have the lowest representation of stem cells, 3 wells of a 24-well plate were seeded with 10,000 cells per well and the number of formed organoids by day 10 was counted for each well (1st generation organoids). Following sequential culturing in organoid conditions, cells showed an increased number of secondary organoids compared to the first generation (Figure 20 and Supplementary Fig. 5), in a similar fashion as observed in sphere cultures (Figure 11), highlighting the enhanced stemness potential of PDAC organoids. Collectively, these data showed that PDAC organoids contain CSCs in a similar level to spheres and are therefore a suitable culture system to model CSCs in vitro.
Figure 20. Representative organoid numbers over one generation (gen.). Data are representative of 1953 primary PDAC PDX-derived in vitro culture (n=2, each performed in triplicate). Error bars, s.d.

3.2 Cancer Stem cell related genes

Using qRT PCR, we determined the relative expression levels of a range of transcription factors **NANOG, BMI1, KLF4, SOX2** and **OCT3/4** which have been recently demonstrated as characteristic of pluripotent stem cells. Data showed an increased expression of these markers in organoid cultures when compared to adherent cultures (Figure 21 and supplementary Fig. 6). Commonly used markers of the stem cell compartment, such as **KLF4, OCT3/4** and **BMI1**, were upregulated compared to sphere cultures.
Figure 21. QPCR analysis of pluripotency-associated genes in organoid and sphere cultures compared to adherent cells of a representative PDX-Tumour, case 1953.

3.3 PDAC organoids express stem cell markers

Using immunofluorescent staining for stem cell markers, we analyzed PDAC organoids for the presence of pancreatic CSCs. We conducted immunohistochemical analyses of various CSC markers, in order to confirm their expression. Paraffin-embedded organoids showed expression of CXCR4 with membrane localization (Figure 22). Data confirm the 1-2% of marker expression observed by flow cytometry (Figure 18).
**Figure 22.** Confocal images of 15 days organoids immunostained for stem cell marker CXCR4 (green) and DAPI (blue). Data are representative of the case 1953.
4 PDAC organoids showed resistance to therapy

Since CSC are believed to be the mediating cell type behind chemoresistance, and since our data show that organoid cells display a more CSC phenotype, we sought to exploit this cell population for the purposes of therapeutic applications. Specifically, we analyzed organoid cultures derived from an index PDX case (1953). We treated adherent cells and organoid cultures with Gemcitabine and Abraxane alone or in combination for 7 days at the end of which we assessed cell viability. Organoids were largely insensitive to these chemotherapeutic drugs, whereas adherent cultures resulted in a viability decrease when treated with a combination of drugs (Figure 23).

![Figure 23](image.png)

**Figure 23.** Representative cell viability comparison between adherent cells (left) and organoid cultures (right) from one PDX-Tumor (1953) following treatment with Gemcitabine and Abraxane. Error bars, s.d.

Unlike 2D cell cultures, 3D systems have recently emerged as advanced drug screening platforms since they more adequately mimic *in vivo* conditions. As depicted in Figure 24, spheres and organoid cultures were incubated with chemotherapeutic drugs for seven days and cell viability was evaluated counting the number of formed structures.
per mL. Our preliminary analysis showed that pancreatic organoids exhibit a similar response to therapeutic drugs as spheres, reflecting their inherent stemness properties.

Figure 24. Representative sphere (top) and organoid (bottom) forming capacity of one primary PDAC cell culture (1953) following treatment with Gemcitabine and Abraxane for 7 days.
DISCUSSION
In light of the still devastating prognosis for PDAC patients, the identification of pancreatic CSCs with exclusive tumorigenicity in 2007 (Hermann et al., 2007) created an entirely new research field spurring renewed hope for the development of novel stem cell-specific targeted therapies. It has been recently demonstrated that 3D models, compared to 2D models, mimic tumour microenvironments better, facilitate the formation of ECM, show a more realistic drug response, exhibit more adequate proliferation rates with more representative cellular morphology. We report conditions for inducing the pancreatic cancer cells to grow as organoids in order to probe their potential values as tool to study CSCs. Previous reports have used mouse and human pancreas tissue to develop organoid cultures of ductal cells, which can be manipulated and transplanted in vivo (Boj et al., 2015; Huch et al., 2013). We modified this approach to develop a 3D culture system that allows the long-term expansion of pancreatic cells from PDX-tumours. Among the typical marker associated with pancreas development, we found that all the cells composed organoids express Cytokeratin 19, confirming their epithelial origin. The stemness potential of cells in organoids was assessed by: (i) expression of stem cell surface markers; (ii) expression of pluripotency-associated genes; (iii) self-renewing ability of neoplastic cells. For the first time, our results clearly demonstrate an enhanced stemness potential of the organoid system compared to other models of PDAC, which include monolayer cell cultures, 3D spheres, and PDXs. In particular, the percentage of neoplastic cells expressing stem cell markers was found to be strikingly higher in organoids than in corresponding PDXs, which makes the former a better platform to study CSCs.

Unlike more traditional in vitro systems, organoids are similar to primary tissue in both their composition and architecture. A recent study reported a method to establish tumour organoids that have histological features consistent with parental adenocarcinoma (Boj et al., 2015). This holds true for our system as we showed that transplantation of organoid cells into immunodeficient mice generated tumours that morphologically resembled primary tissues from which cultures were derived. In addition, we revealed that pancreatic organoids shared similar genetic abnormalities with their corresponding primary tumours. Our data support the use of this technology to model the disease and find new therapeutic targets for PDAC. PDAC is extremely
chemorefractory, with available drugs only giving modest benefit to patients. In a recent study published in 2013 by Von Hoff, it has been shown that combining gemcitabine with abraxane leads to an improvement in survival (Von Hoff et al., 2013). In particular, the survival curves showed a median improvement of 1.8 months and an improvement of 3.4 months at the time point when 25% of the patients were alive. However, even when an initial response to the treatment is observed, tumours often progress. CSCs are believed to be the cause of the observed tumour resistance to the treatment as they can rapidly repopulate tumour mass. Therefore, targeting CSCs might improve cancer treatments. Nevertheless, to achieve these improvements, a key step is to identify and characterize the targets of such treatments – CSCs – in the respective tumour types.

Preclinical testing of candidate drugs in PDAC almost invariably relies on the use of monolayer cell cultures, which are difficult to establish from resected tumours and have been shown to poorly predict responses to treatment. PDXs are considered a better system to predict therapeutic responses in PDAC, while their generation being costly and time consuming. Using an index case, we demonstrated here that PDAC organoids are highly resistant, thereby reflecting inherent stem properties, and exhibit a resistance/sensitivity profile similar to that of corresponding PDX. Our data suggest that human PDAC-organoids represent an important resource for developing personalized treatment.
CONCLUSIONS
We have accumulated compelling evidence establishing PDAC organoids as novel tool for study pancreatic cancer stem cells. Therefore, we conclude:

1. PDAC organoids express typical markers of primary disease.
2. Organoid cells are enriched in stemness-associated genes as compared to adherent cell cultures.
3. Organoids cells showed self-renewal properties *in vitro* as showed the organoid formation capacity assay.
4. Organoid cells are tumorigenic, as we showed with our *in vivo* experiments.
5. Transplantation of PDAC organoids morphologically resembles PDX.
6. Organoids maintain the same genetic profile of the parental tumour.
7. Organoid cultures are more resistant to standard chemotherapy Gemcitabine and Abraxane.

Related to this, the defined nature of the culture conditions means that organoid cultures are ideal for studying stem cells in a 3D environment, which remains a relatively unexplored area for pancreatic cancer. In addition to their role in investigating stem cell biology, 3D organoid cultures promise to be of considerable biomedical utility.
BIBLIOGRAPHY


APPENDIX
Supplementary Figure 1. Flow cytometry analysis of CD44+CD133+ cells from primary PDAC 2636, 140114, and 215 cells cultured as adherent cells or spheres.
**Supplementary Figure 2.** Flow cytometry analysis of autofluorescent content in adherent (left) and sphere cultures (right). Autofluorescent cells are excited with a 488-nm blue laser and best selected as the intersection with filters 530/40 and 580/30.
Supplementary Figure 3. Representative sphere numbers and diameters (µm) for different primary cell culture over one generation (gen.). Data are representative of primary PDAC PDX-derived in vitro cultures (n=3, each performed in triplicate). Error bars, s.d. A, case 140114; B, case 215; C, case 2636.
Supplementary Figure 4. Flow cytometry analysis of autofluorescent content in PDAC organoid cells. Autofluorescent cells are excited with a 488-nm blue laser and best selected as the intersection with filters 530/40 and 580/30.
Supplementary Figure 5. Representative organoid numbers over one generation (gen.). Data are representative of 215, A and 140114, B, primary PDAC PDX-derived in vitro culture (n=2, each performed in triplicate). Error bars, s.d.
Supplementary Figure 6. QPCR analysis of pluripotency-associated genes in organoid and sphere cultures compared to adherent cells of a representative PDX-Tumours (A, case 215; B, case 140114).
Acknowledgements

I dedicate this thesis to my family.

Firstly I would like to thank Prof Aldo Scarpa and Prof Christopher Heeschen for enabling me to pursue such an interesting and exciting PhD project. I would also like to thank Heeschen’s group, especially Dr Marianthi Tatari for her time, encouragement and support, and my work friends, Mingh-sin, Ala, Forough, Claudia, Michele, Eliana and Giona for their friendship and humour. Everyone within the group has been amazingly supportive throughout my PhD and have helped me to start believing in my own abilities as a researcher.

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Balancing my social life with the demands of a PhD has been challenging and so I would like to thank my amazing friends, who have been there for me whenever I needed to talk, eat and DRINK!! I am always amazed at the ability of these wonderful people to make me forget about work stresses and concentrate on the important things in life such as fizzy booze and being silly.

My family have been wonderful throughout my studies, and their love, support and proof-reading services have been second to none.

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