Spring 5-4-2019

# Developing Targeted Therapy against Pancreatic Cancer

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By

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A DISSERTATION

Presented to the Faculty of

The Graduate College in the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Department of Biochemistry and Molecular Biology

Under the Supervision of Dr. Surinder K. Batra

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Omaha, Nebraska

April 11, 2019

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Pancreatic cancer (PC) is one of the most lethal malignancies, and since 53% of patients are diagnosed with the advanced metastatic stage of the disease, handling/targeting this aggressive metastatic cancer becomes a challenge. The present therapy for PC includes gemcitabine, Abraxane, erlotinib, FOLFIRINOX, and their combinations. All of these therapies along with surgical interventions have so far increased the average five-year survival for PC patients to 7.2%. One of the chief contributors for this dismal prognosis of pancreatic cancer is cancer stem cell (CSC) population, which are responsible for the aggressive and refractory nature of the disease. CSCs are a small subpopulation in the tumor, which are held accountable for early metastasis, drug resistance and disease relapse in different cancers including PC [1-3]. Cancer stem cells (CSC) are primary culprits for aggressive metastatic nature of pancreatic cancer, and present therapies fail to target CSC. EGFR family of proteins is vital for CSC self-renewal and maintenance and necessary for PC initiation and progression. In this thesis, we aim to develop a targeted therapy for pancreatic cancer by targeting cancer stem cell population thereby decreasing the metastasis.

In the first part of this thesis, we utilize tissue murine and human origin to develop three-dimensional organoids to be able to evaluate therapy response in them. We show that we successfully generate organoids for mouse pancreatic cancer, normal pancreas, human PDAC samples, and mouse prostate and lungs. We engage PDAC organoids for further studies and show retention and expansion of different subsets of cancer stem cell

population in these organoids. We also standardize a method to evaluate growth and inhibition in established organoids.

In the second part of this thesis, we evaluated a targeted therapy using pan-EGFR inhibitor, Afatinib, aimed at inhibiting Pancreatic CSC and hence abrogate tumor and metastasis. We begin by demonstrating the presence of higher CSC in patients treated with chemotherapy compared to patients with no history of chemotherapy highlighting the presence and role of CSC in PDAC. We then demonstrate the efficacy of treatment with Afatinib alone and in combination with gemcitabine in organoids derived from human PDAC patients. We also show inhibition of primary tumor and metastatic incidence in an orthotopic mouse model of pancreatic cancer by combination therapy of Afatinib and gemcitabine. We also look at changes in the CSC population, KPC organoids and primary tumors in an orthotopic mouse model and find a significant decrease. Upon evaluation of effects of Afatinib on CSC population and KC, KPC organoids we reveal that Afatinib inhibits CSC stemness by downregulating CSC and self-renewal markers. Finally, we provide a mechanism of action for Afatinib and its validation by knockdown studies. Our findings indicate that Afatinib inhibits pancreatic CSC via EGFR/ ERK/ FOXA2/ SOX9 axis and a combination of Afatinib and gemcitabine is an efficient therapy for metastatic pancreatic cancer.

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| | |
|---|---|
| ADCC | Antibody-dependent cellular cytotoxicity |
| AFE | Anterior foregut endoderm |
| ALCAM | Activated leukocyte cell adhesion molecule |
| APACT | Nab-paclitaxel and Gemcitabine vs Gemcitabine Alone as Adjuvant Therapy for Patients with Resected Pancreatic Cancer |
| AR | Androgen receptor |
| ATCC | American Type Culture Collection |
| ATP | Adenosine triphosphate |
| ATRA | All Trans Retinoic Acid |
| BRAF | Serine/threonine-protein kinase B-raf coding gene |
| CAF | Cancer-associated fibroblasts |
| CRP | C-reactive protein |
| CSC | Cancer stem cell |
| CST | Cell Signaling Technology |
| DE | Definitive endoderm |
| DHT | Dihydrotestosteron |
| DMEM | Dulbecco's minimal essential Medium |
| DMSO | Dimethyl sulfoxide |
| ECM | Extra Cellular Matrix |
| ECOG | Eastern Cooperative Oncology Group |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ESA | Epithelium Specific Antigen |
| ESC | Embryonic stem cells |

| | |
|---|---|
| FA | Folinic acid |
| FACS | Fluorescence-activated cell sorting |
| FBS | Fetal bovine Serum |
| FDA | Food and Drug Administration |
| FGF | Fibroblast growth factor |
| FOLFOX | Folinic acid (leucovorin) fluorouracil (5FU) |
| GVAX | GM-CSF (granulocyte-macrophage colony-stimulating factor) secreting vaccine |
| H&E | Hematoxyline and Eosin |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HGF | Hepatocyte Growth Factor |
| HIFU | High- intensity focused ultrasound |
| HLO | Human lung organoids |
| IMPaCT | Individualized Molecular Pancreatic Cancer Therapy |
| IRE | Irreversible electroporation |
| LAK | Lymphokine-activated killer |
| LAPC | Locally advanced unresectable pancreatic cancer |
| MDSC | Myeloid-derived suppressor cell |
| MEC | Mammary epithelial cell |
| MMTV | Mouse mammary tumor virus |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NEOPAC | Neoadjuvant Paclitaxel |
| NSCLC | Non-small cell lung carcinoma |
| OOPD | Office of Orphan Products Development |
| PBS | Phosphate buffer saline |
| PC | Pancreatic cancer |

| | |
|---|---|
| PCNA | Proliferating cell nuclear antigen |
| PCR | Polymerase chain reaction |
| PDAC | Pancreatic ductal adenocarcinoma |
| PSC | Pancreatic stellate cells |
| PCSCs | Pancreatic cancer stem cells |
| QOL | Quality of life |
| QPCR | Quantitative PCR |
| QRTPCR | Quantitative real time PCR |
| RA | Retinoic Acid |
| RECIST | Response evaluation criteria in solid tumors |
| RIPA | Radio-immunoprecipitation assay buffer |
| RNA | Ribonucleic Acid |
| RSPO | R-Spondin1 |
| RTPCR | Real Time PCR |
| SC | Stem Cells |
| SFRO | Sociéte Française de Radiothérapie Oncologique |
| SHH | Sonic hedgehog |
| SMA | Smooth muscle actin |
| SMO | Smoothened homolog precursor |
| SP | Side population |
| SPARC | Secreted protein acidic and rich in cysteine |
| TIL | Tumor-infiltrating lymphocytes |
| USFDA | United States Food and Drug Administration |
| VAFE | Ventral anterior foregut endoderm |
| VEGF | Vascular endothelial growth factor |
| WAP | Whey acidic protein |

| | |
|---|---|
| WNT | Wingless/integrated |

**ACKNOWLEDGMENTS**

I want to express profound gratitude to my advisor, Dr. Surider K Batra, for his continuous support, encouragement, and guidance through my graduate training. His valuable support and encouragement enabled me to complete my work successfully. Am indebted to him for imbuing and teaching me valuable lessons in critical thinking and innovative thought process. His positivity, enthusiasm and his giving nature are awe-inspiring. I would also like to thank Dr. Moorthy Ponnusamy for his supervision and support. He has been a constant source of guidance. I would also like to thank my supervisory committee members Dr. Apar Ganti, Dr. Joyce Solhiem, Dr. Michael. Hollingsworth, Dr. Ram Mahato, Dr. Moorthy P. Pannusamy and Dr. Amar Singh for their support, guidance, and encouragement.

I want to thank the past and the present members of the Batra lab including Dr. Parthasarathy Sheshacharyulu, Dr. Satyanarayana Rachagani, Dr. Surshil Kumar, Dr. Raghupathi Vengoji, Dr. Imayvaramban Lakshmannan, Sanchita Rauth, Ramakrishna Nimmakayala and Kavita Mallya. Thank you for the useful discussions and for sharing your experience with me.

I would also like to thank the University of Nebraska Medical Center, especially the Department of Biochemistry and Molecular Biology for the support and providing an encouraging environment to pursue my graduate studies. I am also very thankful to the graduate school for UNMC graduate studies assistantship and financial support.

Finally, I would like to thank my family and friends for their love, support, and encouragement. I would especially like to thank my parents and my husband for believing in me and helping me. I am also thankful to my parents-in-law for their blessings. I am also grateful to my brothers and sisters for their prayers and

encouragement. I would forever be indebted to my family and my mentors for encouraging me to pursue my interests.

# CHAPTER 1: REVIEW OF THE LITERATURE

## 1.1 Introduction

Pancreatic cancer (PC) accounts for 3% of cancer instances in the United States (US) but claims 7% of all cancer-related deaths. The past 30 years of constant efforts have managed to increase the five-year median survival rates from 3% to 9% [4]. Such dismal patient survival warrants better therapeutic interventions. Pancreatic ductal adenocarcinoma (PDAC) is the most common form/manifestation of PC. Late diagnosis of PDAC, due to the internal location of the organ and lack of distinct symptoms, leaves PC patients with very few therapeutic options. Additionally, PC poses major recalcitrance to therapy due to extensive stroma which prevents drugs from accessing tumor cells and the presence of therapy-resistant cells or cancer stem cells (CSCs). Furthermore, PC is now being proposed as a systemic disease starting from its conception and hence early metastasis warrants development of systemic therapies [5].

Projections indicate that PC will be the second most common cause of death in the USA by 2030 with only non-small cell lung carcinoma (NSCLC) ahead of it [6]. PC patients when diagnosed have limited options for therapy. Over 50% of patients when diagnosed with PC, present a late stage disease with local and distant metastasis [4]. Most of these patients are not suitable for surgical resection, surgery being the only curative treatment available offers a survival of 15-20% [7]. However, most patients are not eligible for surgery. On diagnosis, cancer is staged from one to four on the basis of imaging and biopsies. Stage one and two are often surgically resectable but stage three is locally advanced and surgically unresectable and stage four is distant metastatic cancer.

We saved around 11,000 lives in 2018 out of over 55,000 patients diagnosed with PC. This is where we stand on the road to therapy for PC. This figure although discouraging shows us how much more we can do. There is a lot unknown about the

disease. In this review, we discuss where we stand in the fight against PC, some available therapies and some options that may turn this fight in our favor. We also discuss some issues that need addressing for a better analysis of clinical trial results and future research avenues.

## 1.2 Resectable Pancreatic Cancer

### 1.2.1 Adjuvant Therapy

Surgery is currently the only potentially curative procedure available for PDAC. However more than 90% of patients that undergo surgery show relapse and would die if not supplemented with additional therapy [7]. This is precisely the reason that adjuvant treatment has been the subject for several clinical trials in the last decade. The patients eligible for adjuvant therapy are advised chemotherapy, radiotherapy or a combination (Chemoradiotherapy; CRT) of both. Since the Gastrointestinal Tumor Study Group (GITSG) trial in 1985 many trials have compared different adjuvant therapy regimens and if there is a need for adjuvant therapy [8]. GITSG was a randomized phase 3 trial that enrolled 43 patients and compared the adjuvant therapy of 5 fluorouracil (5FU) chemo with radiotherapy followed by two years of 5FU alone to observation only. The trial was terminated due to poor accrual of patients and increasingly large survival difference between study arms, but a significant difference in average survival of control arm vs. treatment arm (11 months vs. 20 months) could be observed. Although the study only recruited patients with negative resection margins thereby selecting people already prognostically favored, its results did impress upon the survival benefit of adjuvant therapy over no therapy after surgical resection of the tumor. Many studies after that have looked at the benefit of therapy after surgical intervention. Two options often recommended to patients are CRT and chemotherapy alone. An European organization

for research and treatment of cancer (EORTC) multicenter phase 3 randomized trial enrolled 218 patients and compared the benefits of adjuvant CRT versus observation only and found that CRT offers a survival advantage which was not statistically significant (overall survival of 17.1 months in treatment vs. 12.6 months in observation-only arm); [9]. Another study by European Study Group for Pancreatic Cancer (ESPAC), published a multicentral randomized trial (ESPAC1) in 2004 which recruited 289 patients and tried to weigh chemotherapy, CRT and observation only against each other [10]. They compared the 5-year survival rates and found that 5FU-based-CRT offers a survival disadvantage over observation alone (10% versus 20% 5-year survival rates P=0.05). But 5-FU-chemotherapy after R0 or R1 resection showed a survival advantage by increasing 5-year survival to 21% when compared to no chemotherapy which was 8% (P=0.009). This survival benefit was very encouraging since 18 percent of the patients had positive resection margins, which was an exclusion criterion in the GITSG clinical trial [8],[10]. The CRT and hence its negative results in ESPAC1 trial were criticized by the radio-oncologists; on the contrary, similar survival results for CRT were observed in RTOG97-04 trial [11]. RTOG97-04 trial compared CRT with 5FU to CRT with 5FU followed by Gemcitabine and found no survival benefits.

Although ESPAC1 study proved the benefit of 5-FU chemotherapy as adjuvant therapy, out of 289 patients enrolled 237 died by the end of the study. This still means only 12% of enrolled patients survived. Cumulative results of this study put a serious question on the use of CRT as adjuvant therapy, by highlighting the detrimental effects of chemoradiation. Radiation therapy administration delays chemo administration and may also generate a more resistant tumor population if we consider the recent studies published, which claim radiation therapy enriches the cancers stem cell population [12, 13]. These may be the reason behind the overall decrease in 5-year survival with CRT. A small sample size of GITSG study along with results published from EORTC study and

ESPAC1 trial overwhelmed the GITSG study results and favored chemotherapy as the more effective adjuvant therapy. A meta-analysis of randomized adjuvant therapy trials done by Stocken et al. in 2005 compared five trials including ESPAC and concluded that adjuvant therapy reduces the risk of death by 25% which was statistically significant[14]. There was a median survival of 19 months with chemotherapy and 13.5 months without it. Whereas, chemoradiation showed no significant difference in the risk of death with median survivals of 15.8 months with chemoradiation and 15.2 months without. Subgroup analysis revealed chemoradiation was more effective ($p=0.04$) when compared to chemotherapy ($p=0.007$) in patients with a positive resection margin [14]. Over time, multiple studies have compared various adjuvant chemotherapy regimens out of which some notable drugs are gemcitabine and 5FU, a combination of both and FOLFIRINOX (folinic acid, fluorouracil, irinotecan hydrochloride and oxaliplatin). Since its arrival in the scenario of PC therapy in 1996, gemcitabine has taken center stage as a standard of care for treatment of this disease. CONKO-001, a multicentral phase 3 randomized clinical trial compared median disease-free survival, 5-year overall survival and 10-year survival of 368 patients divided into two groups of gemcitabine adjuvant therapy and observation alone [15]. The results depicted that gemcitabine adjuvant therapy led to a median disease-free survival of 13.4 months compared to 6.7 months with observation only. Gemcitabine group also had increased overall 5-year (20.7%) and 10-year (12.2%) survivals compared to control groups of observation only 5-year (10.4%) and 10-year (7.7%) survival rates [15, 16]. Enduringly, ESPAC 3 compared 5FU with folinic acid versus gemcitabine and found no statistical difference in the survival advantage offered by the two drug regimens [17]. The median survival of 5FU + folinic acid was 23.0 months compared 23.6 months with gemcitabine. Median progression-free survival was also 14.1 and 14.3 months respectively. Although gemcitabine alone did not have a significant survival advantage over 5FU+folinic acid, its toxicity profile was

significantly better than later. A retrospective analysis of ESPAC 3 trial suggested a role of ENT1 (equilibrative nucleoside transporter 1, a key mediator for gemcitabine cellular uptake) in stratifying patients for adjuvant administration of gemcitabine [17, 18]. Another prospective study analyzed the prognostic influence of gemcitabine-related genes (hENT1, ribonucleotide reductase subunits (RRM1, RRM2), and deoxycytidine kinase (dCK) ) in 100 patients undergone gemcitabine adjuvant therapy and identified low expression of hENT1 and dCK, and high/moderate levels of RRM1 as negative prognostic factors [19]. These studies suggest stratification of patients before adjuvant therapy administration although further prospective validation is required. Other components of major drug metabolic pathways could be used to refine therapeutic approaches further and generate more personalized treatment regimens. Patient prognosis was also influenced by the ability of the patient to complete all six cycles of chemotherapy [20]. Considering CONKO-001 trial results that established a 6-month course of adjuvant chemotherapy (gemcitabine) as the standard of care for resected PC patients and the positive results of the PA.3 phase III trial [21] demonstrating a survival benefit from the addition of erlotinib to gemcitabine (although in metastatic disease) CONKO-005 trial was designed [22]. The trial revealed no difference in median disease-free survival or median overall survival of gemcitabine and erlotinib combination over gemcitabine alone. Although a trend toward better long-term survival in favor of gemcitabine and erlotinib group was observed (estimated survival after 1, 2, and five years for combination group was 77%, 53%, and 25% v 79%, 54%, and 20% for gemcitabine alone group, respectively). A randomized phase III clinical trial conducted on Japanese population revealed adjuvant therapy of S-1 (combination of tegafur-chemotherapeutic prodrug of 5FU, and chemotherapeutic adjuncts gimeracil (Stabilises 5FU) and oteracil potassium(reduces 5FU activity)) provided a 5-year survival of 44.1% compared to gemcitabine which allowed a 5-year survival of 24.4% [23]. Hence, the

study proposed S-1 as a standard of care for PDAC although the results of this study have not been confirmed in non-Asian populations. A recent trial ESPAC-4 compared gemcitabine monotherapy with gemcitabine plus capecitabine combination therapy in patients with R0 (42% patients) or R1 (60% patients) resected PC. Median overall survival was 28 months for patients in the gemcitabine plus capecitabine group compared with 25·5 months in the gemcitabine group [24, 25]. Consequently, in 2017 the American Society of Clinical Oncology (ASCO) clinical practice guidelines were updated for potentially curable PC. Updated guidelines direct patients with resected PC and no prior preoperative therapy to be offered 6 months of adjuvant chemotherapy in the absence of medical contraindications. In patients with no concerns for toxicity or tolerance, the doublet regimen of gemcitabine and capecitabine should be preferred. Alternatively, monotherapy with gemcitabine or 5-FU plus FA can be offered as an adjuvant treatment initiated within 8 weeks of surgical resection, assuming complete recovery [26]**.** Another important clinical trial named APACT compares doublet therapy of nab-paclitaxel plus gemcitabine with gemcitabine monotherapy, and its results are still pending [27]. In 2018 results of PRODIGE 24/CCTG PA.6 trial were released which showed a large increment in treatment benefit over gemcitabine [28]. PRODIGE 24 was a randomized phase III trial including 493 patients with surgically removed PC. The trial compared mFOLFIRINOX (modified FOLFIRINOX containing oxaliplatin, leucovorin, irinotecan, and fluorouracil) with gemcitabine adjuvant therapies given 3 to 12 weeks after surgery for 6 months. The median disease-free survival and median overall survival observed in the mFOLFIRINOX group (21.6 and 54.4 months) was higher than in the gemcitabine group (12.8 and 35.0 months). At a median follow up patients in mFOLFIRINOX lived a median of 20 months longer. Patients in the mFOLFIRINOX group also exhibited delayed metastasis (median = 30.4 months vs. 17.0 months with gemcitabine) and were cancer-free 9 months longer than those who received

gemcitabine. Overall, mFOLFIRINOX led to more severe symptoms for patients but were mostly manageable as the authors of the study report[28]. The symptoms described for mFOLFIRINOX included diarrhea, nausea, vomiting, and fatigue. The side effects mentioned for gemcitabine included swelling, headache, flu-like symptoms, and low white blood cell counts. Both treatments were reported to cause low levels of white blood cells and fever [28].

### 1.2.2 Neoadjuvant Therapy

Immediate surgery followed by chemotherapy is the current standard of care for PC patients with a resectable disease, but an emerging strategy for resectable, borderline resectable or locally advanced unresectable cancer is neoadjuvant therapy [25]. In this scheme of treatment patients are given radiotherapy, chemotherapy or chemoradiotherapy followed by surgically removing the tumor, also called preoperative therapy [25, 29]. The first randomized clinical trial results for neoadjuvant therapy came in 2015 [25]. Which compared neoadjuvant chemoradiation with gemcitabine/cisplatin and surgery to surgery alone [30]. The trials concluded that the preoperative treatment was safe concerning toxicity and mortality, but the results were not statistically significant. The trial had to be terminated due to slow accrual before it could be completed. Apart from this study, there had been several meta-analyses and few prospective and nonrandomized trials evaluating the benefits of neoadjuvant chemoradiotherapy (mostly gemcitabine-based) but no notable randomized trials [31]. The first randomized, phase III trial (PREOPANC-1) that found that patients who received chemoradiotherapy before pancreatic cancer surgery (preoperative) had better disease-free survival than those who started their treatment with surgical tumor resection, which is the current standard of care, were released in 2018 [29]. The trial enrolled 246 patients eligible for surgical resectable pancreatic cancer who were

randomly assigned to two groups. The first group received immediate surgery and the second group received chemoradiotherapy for ten weeks followed by surgery. Both the treatment regimens were followed by chemotherapy after surgery, with the total amount of chemotherapy given being equal in both groups. Chemotherapy was delivered in two parts in the chemoradiotherapy group which received part of the before surgery and the rest after [29]. The median overall survival and time until pancreatic cancer recurrence was higher for patients receiving preoperative chemoradiotherapy (17.1 and 9.9months) when compared with the ones who underwent immediate surgery (13.7 and 7.9 months). Two years survival rate was also higher with preoperative treatment than with immediate surgery (42% vs. 30%). 72% of patients underwent resection in the immediate-surgery group and 62% in the chemoradiotherapy group [29]. A higher proportion of preoperative treatment group patients saw successful microscopic surgical resection when compared to the immediate surgery group (63% vs. 31%). Among patients with R0 margins, the difference in median survival was greater, 42.1 months with preoperative treatment versus 16.8 months with immediate surgery. FOLFIRINOX chemotherapy or FOLFIRINOX combined with stereotactic body radiation therapy appear promising from other studies and should be tested against preoperative gemcitabine and radiation in a randomized clinical trial [32].

Another multicenter, randomized controlled phase III trial called NorPACT- 1 investigates benefits of neoadjuvant chemotherapy based on FOLFIRINOX over surgery with both groups receiving adjuvant chemotherapy with gemcitabine and capecitabine in 90 patients [33]. The trial was organized by the Norwegian Gastrointestinal Cancer Group for Hepato-Pancreato-Biliary cancer for resectable cancer of the pancreatic head to decrease early mortality (within one year) in resected patients. Another notable multicenter randomized phase III trial designed to explore the efficacy of neoadjuvant chemotherapy is NEOPAC trial [33]. In patients with resectable

pancreatic adenocarcinoma in the head of the pancreas were randomized to surgery followed by adjuvant gemcitabine for six months or neoadjuvant gemcitabine and oxaliplatin followed by surgery and the same adjuvant treatment. Neoadjuvant chemotherapy would be given four times every two weeks [33].

Neoadjuvant chemoradiation is also being evaluated for providing substantial local control of borderline resectable pancreatic cancer and prolong patient survival. An open-label, multicentre, prospective phase II trial that was designed to assess S-1 chemotherapy with radiotherapy as neoadjuvant treatment [34]. Participants would receive S-1 and concurrent radiotherapy, with surgery after 3-8 weeks which will last 36-month period with a minimum 24-month follow-up [34].

## 1.3 Nonresectable disease

### 1.3.1 Locally advanced unresectable pancreatic cancer (LAPC)

More than half of the patients that present with pancreatic cancer are diagnosed at the LAPC stage and have a discouraging survival rate of less than 5% [25]. These groups of patients depict local invasion of adjacent structures, generally vascular and are challenging to treat. Unlike resectable PC, LAPC patients rarely undergo resection with a curative intent instead of local control, symptom management and quality of life are the primary goals of the therapy [25].

Initial systemic (induction) chemotherapy with combination regimens is usually recommended for most patients who can tolerate aggressive therapy. There is conflicting evidence for chemoradiotherapy versus gemcitabine chemotherapy alone [25]. ECOG (Eastern Cooperative Oncology Group) 42201 trial results in favor chemoradiotherapy whereas 2000‐01 Fédération Francophone de Cancérologie Digestive (FFCD)/Sociéte Française de Radiothérapie Oncologique (SFRO) trial favors

gemcitabine chemotherapy alone [35, 36]. No standard therapy is proven to be clinically superior to others, and hence an informed decision has to be made by the physicians considering risks and benefits for the patients. Therapies recommended for metastatic patients are often referred to due to lack of evidence for LAPC.

Since 1997 till 2011 gemcitabine was considered standard of care for patients with unresectable pancreatic cancer. A definitive clinical trial assessing Gemcitabine versus 5 FU as first-line treatments for patients with locally advanced pancreatic cancer was conducted [37]. Results from this trial established that gemcitabine improves disease-related symptoms and survival in patients with pancreas cancer (5.6 vs. 4.4 months) [37]. Although the overall survival increment was small, the 12 months survival rate was improved significantly (18% for gemcitabine vs. 2% for 5-FU patients). Many trails since have worked on improving the overall survival and quality of life for patients with advanced pancreatic cancer by adding one or more drugs to gemcitabine and exploring different schedules (fix dose rate) of its administration. None of the trials resulted in clinically appreciable improvement in patient survival over gemcitabine till 2011, PRODIGE4/ACCORD11 trial which revealed FOLFIRINOX, a combination of 4 drugs (irinotecan, oxaliplatin, folinic acid, and 5-fluorouracil) showed a moderate improvement in overall survival of patients [28]. Local therapies for the management of tumors are recommended if the patients do not show a metastatic disease after induction chemotherapy. Several Local, regional therapies have been tested for LAPC management including irreversible electroporation (IRE), radiofrequency ablation (RFA), stereotactic body radiation (SBRT), high- intensity focused ultrasound (HIFU) [38]. RFA and SBRT are the best-studied modalities, and SBRT is the only treatment that has resulted in the quality of life improvements in patients with LAPC. SBRT has been studied with varying guiding techniques and radiation doses applied, and morbidity has been reported in up to 25% of patients [38]. SBRT minimizes the disruption in

chemotherapy since higher doses of radiation is given in shorter durations in contrast to radiotherapy. ASCO included SBRT in their recommendations for LAPC in 2016 [39]. Chemoradiotherapy or SBRT may be offered as an alternative to continuing chemotherapy alone for any patient with LAPC. CRT or SBRT may also be offered to patients who show local disease progression or toxicities after induction chemotherapy [38]. A short course of palliative radiotherapy (5 to 10 treatments) may be offered to patients with LAPC who have severe GI symptoms. Prolonged survival, better symptomatic management, and tumor regression as a result of local ablative therapies, has been reported in several studies [38]. However, utilization of local treatments should undergo further evaluation since approximately 30% to 50% of patients presenting with LAPC have evidence of metastatic disease within three months. Owing to the lack of RCTs conclusive data supporting local ablative therapies for LAPC is sparse [38].

### 1.3.2 Advanced metastatic pancreatic cancer

Gemcitabine became the standard of care and choice for first-line therapy against advanced metastatic pancreatic cancer since the Burris et al. trial in 1997 [37]. Other Phase III trials since then evaluating single-agent gemcitabine monotherapy for advanced pancreatic cancer yielded a median survival of 5.0 to 7.2 months for patients [40]. In 2006, a Randomized phase III trial compared gemcitabine plus cisplatin with gemcitabine alone in patients with advanced PC. The trial included 195 patients and the doublet therapy arm showed a prolonged progression-free survival compared to gemcitabine monotherapy (5.3 months v 3.1 months). Median overall survival was also enhanced in patients treated with a combination of gemcitabine and cisplatin as compared with the Gemcitabine (7.5 v 6.0 months). However, it was not statistically significant. The results from this trial favored the combination of gemcitabine with cisplatin over gemcitabine monotherapy [40]. Another trial from the Swiss Group for

Clinical Cancer Research and the Central European Cooperative Oncology Group compared Gemcitabine plus capecitabine combination therapy with gemcitabine monotherapy in advanced pancreatic cancer in a randomized, multicenter, phase III trial [41]. Median overall survival was improved although not significantly with the addition of capecitabine by an average of 1.2 months (8.4 months in Gemcitabine and capecitabine arm vs. 7.2 months in Gemcitabine arm). Further analysis of the trial reviled that the combination therapy was significantly beneficial for patients with good performance score (KPS of 90 to 100) with median overall survival being 10.1 months for combination group versus 7.4 months for Gemcitabine group. Additionally, a meta-analysis of randomized clinical trials indicated a significant survival benefit to patients when Gemcitabine base therapies combined with either platinum analogs or fluoropyrimidines. Their subgroup analysis that included 38% of all patients included in the meta-analysis, Gemcitabine-based combination therapies were beneficial for patients with a good performance score but not for patients with a poor performance score [42]. Around the same time phase II studies showing survival advantage and benefits of single-agent Irinotecan, and oxaliplatin combination with fluorouracil came out [43, 44]. Considering synergistic observed between Oxaliplatin and irinotecan in vivo, an open-label phase I study assessed the triple combination of oxaliplatin plus irinotecan plus leucovorin/5-fluorouracil in patients with advanced solid tumors and saw encouraging results. This prompted a phase II and later Phase III trial comparing a combination chemotherapy regimen of FOLFIRINOX (oxaliplatin, irinotecan, fluorouracil, and leucovorin) with gemcitabine as first-line therapy in patients with metastatic pancreatic cancer (PRODIGE 4/ACCORD 11) [28]. The median overall survival and progression-free survival were both enhanced FOLFIRINOX group versus gemcitabine group(11.1 vs. 6.8 months and 6.4 vs. 3.3 months, respectively) [28]. The trial reported FOLFIRINOX to be associated with a survival advantage but increased toxicity. Another phase III MPACT trial

compared *nab*-paclitaxel and gemcitabine combination therapy versus gemcitabine alone in patients with metastatic pancreatic cancer [28]. The study demonstrated a significant survival advantage of *nab*-paclitaxel and gemcitabine (8.5 vs. 6.7 months). Progression-free survival was also improved with the combination therapy over gemcitabine monotherapy (5.5 vs. 3.7 months). After the results of MPACT and PRODIGE trials, ASCO guidelines were updated to include the proposed therapies. Gemcitabine plus NAB-paclitaxel was recommended for patients with Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0 or 1, relatively favorable comorbidity profile, and tolerability for relatively aggressive medical therapy [28]. Whereas, FOLFIRINOX (leucovorin, fluorouracil, irinotecan, and oxaliplatin) was recommended for patients who have an ECOG PS 0 or 1, favorable comorbidity profile, and can tolerate aggressive medical therapy, with access to chemotherapy port and infusion pump management services. On the other hand, patients with metastatic pancreatic cancer ECOG performance status, two are referred gemcitabine monotherapy according to the ASCO Clinical Practice Guidelines updated in 2018 [45].

Another approach is to capitalize on the hypoxic environment in pancreatic cancer. TH-302 is a prodrug of cytotoxin Bromo-isophosphoramide mustard (Br-IPM) and is hypoxia-activated. Evofosamide was tested in a randomized phase II clinical trial in a combination of gemcitabine versus gemcitabine alone and resulted in a significant increment in Overall survival from 3.6 months (Gemcitabine alone) to 6 months [46]. Despite encouraging results from prior trials, evofosamide failed to significantly improve overall survival in combination with gemcitabine in previously untreated patients with unresectable locally advanced or metastatic PDAC in the randomized phase III MAESTRO trial **[47]**. The trial reported an increase in median progression-free survival in the combination group versus gemcitabine alone group (5.5 months versus 3.7

months) encouraging further thought into the therapy. The trial results were mostly disappointing except for Asian patients. Among 123 Asian patients, median OS of 12.0 months could be achieved by the addition of evofosamide to gemcitabine versus 8.5 months with placebo/gemcitabine [47]. This highlights how population diversity may affect results in a trial and the need for therapy regimens to be tested for diverse groups separately.

### 1.3.3 Second-line therapy

Most pancreatic cancers are refractory to front line therapies and hence have a dismal prognosis. The median overall survival remains less than 12 months despite the available front-line therapies warranting development of potent second-line therapies. Despite 16-18% of patients undergoing a second line therapy, there are very few phase III randomized clinical trials available for second-line treatment for metastatic pancreatic cancer. The first phase III study came from CONKO-1 study group comparing best supportive care with oxaliplatin, folinic acid (FA) and 5- fluorouracil (FU) and best supportive care in patients (BSC) not responding to gemcitabine therapy [48]. The trial had to be terminated after 46 patients due to insufficient patient accrual instead of planned 165 patients. Median second-line survival was 4.82 months for oxaliplatin, FA and FU (OFF) treatment and 2.30 months with best supportive care alone. Median overall survival for first-line therapy with gemcitabine followed by OFF was 9.09 and BSC was 7.90 months. Despite being terminated prematurely, this randomized trial provided the first evidence of second-line chemotherapy being superior to best supportive care alone for metastatic pancreatic cancer patients not responding to gemcitabine [48]. After the proven benefit of second-line therapy the follow up CONKO-003 trial compare the second-line OFF treatment versus FF alone for gemcitabine-refractory pancreatic cancer [49]. The study included 168 patients refractory to first-line

gemcitabine therapy who were randomly assigned to FF or OFF. The median overall survival and progression-free survival in the OFF group was 5.9 and 2.9 months, whereas the FF group was 3.3 and 2 months [49]. This significant improvement by addition of oxaliplatin in patient survival was without any clinically relevant enhanced toxicities [49].

PDAC is characterized by an extensive desmoplastic reaction, which complicates drug delivery and access to the tumor. To circumvent the desmoplasia novel formulations and drug delivery mechanisms are being tested. This search was encouraged by improved survival data seen in the NAPOLI-1 study [50]. In NAPOLI-1 trial a combination therapy of nanoliposomal irinotecan (MM-398) plus fluorouracil and folinic acid was tested in patients with advanced metastatic PC and a prior history of gemcitabine-based therapy, against monotherapy with nanoliposomal irinotecan or fluorouracil and folinic acid [50]. Although the median overall survival did not differ much between the monotherapies (nanoliposomal irinotecan4.9 months and fluorouracil and folinic acid 4.2 months), a combination of nanoliposome irinotecan with fluorouracil and folinic acid extended the overall survival to 6.1 months. The survival benefits were observed with a manageable safety profile. A follow-up report from the NAPOLI-1 study reported that overall survival benefits were maintained with no safety concerns. One-year overall survival rates were estimated to be 26% with the combination therapy of MM-398 with fluorouracil and folinic acid and 16% with fluorouracil and folinic acid [50].

There are very few studies testing second-line therapy after first-line therapy of FOLFIRINOX. Most available trials describe second-line therapy options after a first line treatment history of gemcitabine. A retrospective study of patients with advanced pancreatic cancer treated with FOLFIRINOX first-line therapy followed by either doublet

or single agent as second-line therapy reported better overall survival for single-agent chemotherapy [51]. On the contrary, an AGEO prospective multicenter cohort reported a median survival of 18 months from the start of first-line therapy of FOLFIRINOX and a second line therapy of nab-paclitaxel plus gemcitabine. Second-line therapy with nab-paclitaxel plus gemcitabine could achieve an overall survival of 8.8 months and median progression-free survival of 5.1 months [52].

Although new studies have reported a survival benefit from second-line therapy, in patients with good performance status further research is needed to device better second-line treatment. In 2018, ASCO updated recommendations for second-line treatment of advanced pancreatic cancer to include gemcitabine plus nanoparticle albumin-bound paclitaxel (nab-paclitaxel) as second-line therapy for patients with first-line treatment with FOLFIRINOX and ECOG PS of 0 to 1 [45]. The recommendations further suggest options of Fluorouracil plus oxaliplatin, irinotecan, or nanoliposomal irinotecan as second-line therapy for patients with a history of first-line treatment with gemcitabine plus nab-paclitaxel, ECOG PS of 0 to 1, and access to chemotherapy port and infusion pump management services. Single-agent therapies of gemcitabine or fluorouracil are recommended for patients with ECOG PS of 2 and who can not tolerate more aggressive treatments [45].

### 1.4 Targeted therapies against PDAC

Therapies targeting specific signaling pathways or molecules associated with cancer growth or survival are designed to target cancer cells, leaving healthy cells relatively unharmed. Several targeting agents have been evaluated over the years against PC alone and in combination with chemotherapeutic drugs. So far, none of the targeted therapies has yielded a significant benefit to patient survival except EGFR family-

targeting erlotinib, which has produced a moderate survival benefit. In the past few years, many therapies targeting important signaling cascades in PC have failed in randomized clinical trials with gemcitabine-based chemotherapy [25].

Targeted therapies have still to show a significant survival benefit for a collective patient population for pancreatic cancer, but patient stratification may improve therapy response. In a randomized phase III trial which compared gemcitabine plus erlotinib targeted therapy with gemcitabine [53] overall survival was significantly prolonged to 6.24 months v 5.91 months for gemcitabine. Progression-free survival and one-year survival was also higher with erlotinib plus gemcitabine. Further analysis revealed that a subgroup of patients who developed skin rash upon erlotinib treatment had a median survival of 12 months [53]. A skin rash can be generally considered as a positive predictive marker for response to anti- EGFR therapy across tumor entities. However, the molecular mechanism behind this phenomenon is still unknown. Understandably, high molecular heterogeneity and extensive and complex stromal and inflammatory components may be the reason behind the failure of targeted therapies in an unselected population suffering from advanced PDAC, since molecular profile and stromal content affect the half-life, accessibility, and metabolism of the administered therapeutic agent.

### 1.5 Targeting tumor microenvironment

The pancreatic tumor microenvironment is characterized by immense stroma, hypoxia, and hypovascularization. The nature of the tumor microenvironment can be a guiding factor for appropriate therapy regimen. Antiangiogenic targeted therapies have not been beneficial, such as the vascular endothelial growth factor (VEGF) inhibitors, and multikinase inhibitors with antiangiogenic activity [25]. This may be attributed to the extensive stroma and hypovascular environment of pancreatic tumors. Exploiting the stroma to enhance chemotherapeutic drug delivery is an attractive prospect. After

patient survival was significantly improved with gemcitabine plus nab-paclitaxel over gemcitabine monotherapy Secreted protein acidic and rich in cysteine (SPARC) was proposed to enrich the concentration of nab-paclitaxel in the pancreatic cancer microenvironment [25]. SPARC is a cancer-associated fibroblast (CAFs) produced matricellular protein known to bind albumin and hence could enhance binding of nab-paclitaxel to tumors and increase antitumor activity. However, no association to validate that theory could be observed [54].

The pancreatic cancer stroma contains several cell types, including inflammatory cells, blood vessels, nerve cells, and CAFs. Pancreatic stellate cells (PSCs) are activated fibroblasts and play a vital role in developing a pancreatic tumor[55]. Hence, PSCs are emerging therapeutic targets. Treatment with Vitamin D ligand calcipotriol could reverse activated PSCs to a quiescent state in a preclinical study. Since PSCs express high levels of vitamin D receptor, their inhibition led to decreased fibrosis and enhanced tumor uptake of gemcitabine [56]. A phase 1b study is currently underway testing a combination of two chemotherapy drugs gemcitabine and nab-paclitaxel with all trans retinoic acid (ATRA) in patients with locally advanced or metastatic pancreatic cancer [57]. Emerging preclinical studies have shown that a derivative of Vitamin A, ATRA, has the potential to remodel or suppress stroma to enhance antitumor efficacy of drugs [57].

The stroma also comprises a variety of extracellular matrix, components, collagen, fibronectin, laminin, and hyaluronic acid. Hyaluronic acid readily binds fluid and increases intratumoral pressure to collapse vasculature and hence reduce drug uptake — Pegylated recombinant human hyaluronidase (PEGPH20) in an experimental drug that breaks up hyaluronic acid [58]. In a recent phase, II study the addition of PEGPH20 to nab-paclitaxel/gemcitabine improved progression-free survival in patients with

metastatic pancreatic cancer with no prior treatment. Although the trial was briefly interrupted due to thromboembolic events in the PEGPH20 group, the risk of thromboembolic events was reduced in all patients with the use of enoxaparin prophylaxis although the median overall survival was not improved (9.6 vs. 9.2 months) amongst patients [58]. A retrospective analysis of the study revealed benefit to patients with hyaluronan-high tumors (overall survival of 11.5 vs. 8.5 months, Median progression-free survival of 9.2 months vs. 5.2 months) [58].

However, extreme caution should be undertaken while targeting PDAC stroma. Results from mouse models and in-vitro studies are often not replicated in patient trials. In preclinical studies in mouse models inhibition of Hedgehog signaling with Smoothened homolog (SMO) inhibitor IPI-926 enhanced gemcitabine delivery to pancreatic tumors by inhibiting myofibroblast growth, collagen deposition, and Tumour vascularity [59]. However, the combination therapy of IPI-926 and gemcitabine failed improved survival over gemcitabine monotherapy and led to the termination of the trial due to the progression of disease in the treatment arm [59]. It was demonstrated later that longer-term IPI-926 administration did inhibit stroma and increase vascularization but enhanced tumor growth and metastasis in a preclinical mouse model of pancreatic cancer. Thus, the stroma seems to have dual roles of protecting against tumor progression and impairing drug delivery to the tumor [60]. Targeting it is a double edge sword, and more research is required to devise strategies to target the stroma and avoid compromising its protective role optimally.

The hypoxic nature of the pancreatic tumor microenvironment offers a therapeutic window to target cancer cells selectively. Evofosamide failed to improve overall survival with statistical significance in an unselected population but was

significantly beneficial for Asian patients [47]. The study is another example of how patient stratification can alter clinical trial result interpretation.

### 1.6 Immunotherapy

Immune response plays dual-opposing roles in cancer biology. On the one hand, it regularly filters and eliminates cancerous cells, and on the other hand, it can potentiate tumor growth by suppressing host immune response. The immune suppressive microenvironment is one of the critical hallmarks of PC characterized by upregulation of immune suppressive module that helps tumor cells to evade and silence the host's immune response [61]. Immune suppression is achieved either directly or indirectly by cancer cells. The mechanism used by cancer cells to bypass immune clearance includes directly expressing Teff cell inhibitory proteins on the cell surface and maintaining immunoinhibitory cytokine profile (CXCR2, IL8) via MDSCs and T cells indirectly [25].

A long-acting pegylated form of recombinant IL10, called pegilodecakin, stimulates the expansion of cytotoxic CD8 T cells and enhances their tumor infiltration [62]. The increased infiltration of CD8-positive T cells has been shown to improve patient prognosis. Hence exerts anticancer effects. Results from a previous phase I/Ib clinical trial demonstrated a good safety/tolerability profile and sustained antitumor effects for pegilodecakin in several cancers including pancreatic cancer [62]. A combination regimen of pegilodecakin and FOLFOX yielded a median progression-free and overall survival of 3.5 months and 10.2 months respectively [62]. The one-year survival rate for the combination regimen was 47% with a disease control rate of 79%, and a complete response rate of 11%. Following the encouraging results, a randomized clinical trial SEQUOIA is underway comparing a combination of pegilodecakin and FOLFOX to FOLFOX alone as second-line therapy in patients with pancreatic ductal adenocarcinoma after tumor progression during or following a gemcitabine-based

therapy [62]. Pegilodecakin was granted the designation of an Orphan Drug for the treatment of pancreatic cancer by the U.S. Food and Drug Administration (FDA) and European Commission. US FDA also granted Fast Track designation for pegilodecakin plus FOLFOX as a second-line therapy against PC.

Another new notable drug is immune modulating multicomponent complex, YS-ON-001. It is an emerging immune-oncology product with a broad spectrum of immune modulating actions like activation of natural killer cells, regulation of macrophage polarization, induction of antitumor cytokines, and suppression of regulatory T cells. Animal studies with YS-ON-001 in multiple solid tumors, including, pancreatic cancers have demonstrated antitumor efficacy and good safety of the product. The FDA Office of Orphan Products Development (OOPD) has granted YS-ON-001 an Orphan Drug designation with the potential to provide safe and effective treatment, diagnosis, or prevention of rare diseases/disorders that affect fewer than 200,000 people in USA.

### 1.6.1 Checkpoint blockade

Checkpoint inhibitors or inhibitors targeting cell surface proteins that inhibit T effector cells have been subject of several studies trying to target PDAC, but none has yielded significant clinical benefit. Only phase I-II trials have published results with combination therapy including PD-1, PD-L1, and CTLA-4 inhibitors in PC patients and only reported only a small improvement over clinical trials of single-agent checkpoint blockade. α-CTLA4 is a CD28 homolog and is expressed on T effector and regulatory cells and was the first checkpoint inhibitor to be targeted in phase II clinical trial with ipilimumab which did not yield an improvement in overall patient survival [63]. Ensuingly, several clinical trials utilized ipilimumab in combination with chemotherapy and immune stimulators like GVAX [64]. Additionally, a phase one trial evaluated dose escalation of anti CTLA4

antibody with gemcitabine chemotherapy and reported safe and tolerable patient profile [65]. These studies encouraged further clinical trials testing alternative combinations and doses of anti CTLA4 therapy, most of which have not posted results yet.

Several phase I-II trials have since demonstrated the feasibility and potential of improvement by adding checkpoint inhibitors to gemcitabine and/or nab-paclitaxel in the first- and second-line treatment settings in advanced pancreatic cancer [66-68]. Available evidence suggests that combination chemotherapy and checkpoint blockade may be more efficacious in a treatment-naïve setting compared to the second-line therapy for patients with advanced PDAC with increasing toxicity [66, 68]. Most notable results have been from a quadruplet regimen of gemcitabine plus nab-paclitaxel plus durvalumab plus tremelimumab in treatment-naïve metastatic PDAC in a phase II trial [67]. Disease control rate was reported as 100% with 6-month survival rate as 80%, and the quadruple treatment was well tolerated. Pembrolizumab has also been tested in neoadjuvant setting with chemoradiation for borderline resectable PDAC in phase Ib/II trial [69]. A bifunctional anti-PD-L1 and TGFβ receptor II fusion protein have also shown tolerability in PDAC patients suggesting the possibility of multitargeted fusion constructs involving checkpoint blockade [70]. Selecting PDAC patients earlier in the treatment course will also allow patients to develop a full-scale immune response with less immune tolerance and more time to recover from immune-related adverse events.

Several ongoing clinical trials are investigating combination therapies including checkpoint blockade in the perioperative setting to examine the efficacy of immuno-oncology approach in the non-metastatic setting. Although CTLA4 and PD1/PDL1 are the most well-studied immune checkpoints, other immunoinhibitory molecules such as LAG-3, TIM-3, and A2AR, are also being considered for immunotherapy in clinical trials [71]. Immune cells express a CD40 at their cell surface, and its binding to the CD40

ligand expressed on $CD4^+$ helper T cells results in the activation of APCs (Diehl L et al. 1999). A phase I clinical trial evaluating CD40 agonist mAb therapy in combination with gemcitabine produced a tumor response in 19% of patients with unresectable chemotherapy naive pancreatic cancer [72]. Tumor biopsies from these patients showed limited tumor-infiltrating lymphocytes and abundant tumor-infiltrating macrophages. Currently, a phase I clinical trial is underway evaluating R07009789 (a CD40 agonist mAb) combination with gemcitabine and nab-paclitaxel for patients with resectable pancreatic cancer.

However, there are no large-scale randomized phase III trials comparing combination therapies with checkpoint inhibitors with the current standard of care in PDAC. Nevertheless, ongoing studies lay some path for future studies for combination regimens integrating checkpoint blockade.

### 1.6.2 Cancer vaccine

Cancer vaccines are aimed at enabling the host to generate a T cell-mediated immune response by augmenting antigen presentation. To elaborate onco-antigens are provided as vaccines, which when encountered by the host's antigen presenting cells are degraded and displayed on their cell surface and presented to effector T cells and memory T cells. This primes the T cells to launch a cytotoxic T cell response and developing anti-tumor immunity [72]. Some relatively well-studied antigens common in PCs and potential targets for vaccine developments include MUC-1 (Mucin-1), MUC4 (Mucin-4), mutated KRAS and CEA (Carcinoembryonic antigen) [73, 74].

GVAX is whole cell vaccine composed of irradiated autologous pancreatic cancer cells genetically modified to secrete GM-CSF (granulocyte-macrophage colony stimulating factor). GM-CSF secreted by these PC cells potentially stimulates dendritic cells (DCs)

to prime both B- and T-cells against cancer cells. GM-CSF also enables enhanced antibody-dependent cellular cytotoxicity (ADCC) and potentiated LAK (lymphokine-activated killer) cell toxicity through IL2 secretion. Ipilimumab combination with GM-CSF cell-based vaccine (GVAX) in a study of 30 patients resulted in enhanced median overall survival and one-year survival over ipilimumab alone (3.6 vs. 5.7 months and 7% vs. 27%, respectively) [75]. It is notable that higher levels of mesothelin-specific $CD8^+$ T cells were observed in patients in both treatment arms indicating an improved anti-cancer T-cell response and had prolonged overall survival. A potential caveat to checkpoint inhibitor therapy is lack of cytotoxic infiltrating cells and hence supplementing it with GVAX made sense. GVAX is currently being studied in patients with resectable pancreatic cancer in phase I/II clinical trial with or without PD-1 binding monoclonal antibody nivolumab (NCT02451982; clinicaltrials.gov).

RAS mutations are the most common genetic mutations in pancreatic cancer. A vaccine called TG01 developed by Targovax contains seven mutated RAS peptides, which when injected into patients could potentiate programming of T cells to launch a cytotoxic immune response. A phase I/II clinical trial in Norway tested this peptide-based RAS mutation targeting cancer vaccine in pancreatic cancer patients undergoing chemotherapy. The trial included 32 patients and showed TG01 allowed an astounding survival advantage over 4.6 months, the median survival time after diagnosis for pancreatic cancer patients in Europe and allowed a median overall survival of just under three years. While these results are promising it will be interesting to explore the potential of TG01 when combined with checkpoint inhibitors.

Immune modulation of tumor stroma

CAFs are active members of PC and facilitate tumor suppressive environment. CXCL12 is abundant in pancreatic cancer microenvironment, whereas CXCR4 is expressed on

cancer and endothelial cells. CXCL12 also acts as a chemoattractant for hematopoietic cells including macrophages and T cells since they express CXCR4.FAP+ stromal cells produce CXCL12( C-X-C motif chemokine ligand 12 ) which binds to CXCR4 ( C-X-C chemokine receptor type 4 ) and functions to promote cellular chemotaxis suggesting a mechanism of tumor-stromal cross-talk [76]. Although, depleting FAP+CAFs have shown synergy with a vaccine based or immune checkpoint-based immunotherapies in mouse models it is not advisable to, pan-target FAP+ fibroblasts since they are vital for normal homeostasis and present unanimously in the human body [77, 78]. Mouse studies have shown treatment with AMD3100 (an antagonist anti-CXCR4 mAb) lead to T-cell mediated reduction in tumor growth [77]. AMD3100, commercially named Mozobil, is being evaluated in phase I clinical trial for its potential to enhance T-cell infiltration in the tumor microenvironment of patients with advanced pancreatic cancer.

### 1.7 Precision medicine

Pancreatic cancer is a difficult disease to treat and diagnose principally due to late diagnosis and inter and intra-tumor heterogeneity. Early diagnosis of PC can enable better-informed decisions specific for stage and subtype of PC. The most reliable blood test for pancreatic cancer is a serum level of CA19-9 (carbohydrate antigen 19-9) higher than 37 U/ml. This test is 80.3% sensitive and 80.2% specific in detecting pancreatic cancer from healthy patients. CA19-9 level can also differentiate between benign and malignant pancreatic cancer by the specificity of 82.8% and a sensitivity of 78.2% [79]. However, the minimum requirement for sensitivity and specificity for an early detection test to improve patient survival and affordability if 88% and 85%. Efficient early detection of pancreatic cancer can enhance patient survival by 30-40% [6].

In an attempt to allow earlier and more accurate detection of PC a recent study proposed levels of glypican-1on exosomes isolated from patient plasma. The test set up a cut of at 7.6% glypican-1-positive exosomes and detection of benign or malignant pancreatic cancer from healthy patients reported in the study with astounding sensitivity and specificity of 100% [80]. Although cancer-specific exosome isolation avoids contamination by noncancer proteins, exosome isolation from patients is difficult in clinical practice. Genetic analysis of cell-free DNA from patient plasma is more feasible now due to the advent of next-generation sequencing on cell-free media. A recent study reported the diagnostic specificity of 100% and a sensitivity of 92.3% by analyzing 54 genes concomitantly in patient samples [81].If these studies are replicated in more extensive studies a more precise treatment regimen could be designed to allow better patient survival.

Owing to tumor heterogeneity and limited prevalence of distinct mutations in individual patients patient subgrouping is difficult. An amalgamation of high-throughput genomic technologies and advanced system biology algorithms has the potential to utilize genomic features of pancreatic cancer and its microenvironment to facilitate the development of treatment protocols specific to patients. Last decade saw multiple studies subgrouping patient population resulting in two subtypes that had clinical prognostic relevance defined as classic and basal-like. The basal-like subtype of PC was associated with reduced median survival time, more activated stroma and increased expression of genes such as *ITGAM*, *CCL13* and *CCL18 on marcorphages*, and members of the *SPARC*, *WNT* and *MMP* families [82]. The study utilized microarray data for subgrouping and suggested that an RNA-derived signature characterizes the Tumour better than the traditionally employed somatic mutations.

The first clinical trial evaluating personalized medicine over the standard of care gemcitabine in PC was IMPaCT (Individualized Molecular Pancreatic Cancer

Therapy) trial [83]. Patient subgroups that received targeted therapies based on tumor mutations were; mutated homologous recombination and DNA damage repair genes (*BRCA1*, *BRCA2*, *PALB2* or *ATM*); amplified *ERBB2;* absence of KRAS mutations. None of the patients eligible for targeted therapy could be successfully treated in this study. UK Precision-Panc trial and the University of Toronto-initiated COMPASS trial (NCT02750657) are other similar studies utilizing sequencing data to define a patient population with deficient DNA repair mechanisms that could benefit from platinum-based chemotherapy. The feasibility of this method of detection is questionable due to the requirement of highly cellular tumors so the results would have to be critically analyzed.

Despite the dearth of clinical trials supporting personalized therapy abundant treatment options are available in PC to define treatment strategies specific to tumors. Evidence suggests patients with high tumor expression of peroxisomal acyl-CoA oxidase one could benefit from tyrosine kinase inhibitor masitinib and gemcitabine [84]. Similarly, combination therapy with ruxolitinib (Janus kinase 1(JAK1)–JAK2 inhibitor) and capecitabine may favor patients with systemic inflammation depicted by elevated serum C-reactive protein (CRP) levels [85]. Another new promising study reported high tumor levels of hyaluronic acid to suggest a potential benefit from pegylated recombinant human hyaluronidase-based therapy [86] and lead to a biomarker-enriched phase III trial. Similarly, ENT1 and BRAF mutations (not KRAS mutations) expression was found to predict response with gemcitabine [18] and serine/threonine-protein kinase BRAF inhibitors respectively. A recent study evaluated 109 patient biopsies from PDAC patients and reported 22% of PC patients had high microsatellite instability (MSI-H). Considering prognostic relevance of MSI in colorectal cancer Americal Society of Cancer suggests Routine testing for dMMR or MSI-H to find patients for checkpoint inhibitor therapy or PD-1 immune checkpoint inhibitor pembrolizumab as second-line therapy.

The advent of organoids cultures has generated another fabulous platform for the development of personized therapy. Pancreatic tumor organoid cultures recapitulate the full spectrum of disease progression when transplanted orthotopically in syngeneic mice. Tumor organoids represent tumor heterogeneity and maintains cancer stem cells better than 2D culture and retain cell plasticities and epigenetic changes that reflect different stages of PDAC progression. Their utilization for drug screening is still in the nascent stage, and more studies are required to analyze if drug response is replicated in this model as other models of PC.

Precision medicine approaches seem attractive and promising with the potential to facilitate the development of targeted therapies against specific mutation profiles of tumors taking into account the drug delivery, drug metabolism and adverse effects for a particular patient. The low tumor incidence and higher variations in targetable genes along with an implicit sampling bias due to tumor heterogeneity are possible limitations to the development of personalized medicine.

### 1.8 Evaluating Therapy response with a grain of salt

Most of our preclinical studies utilize traditional cell line cultures, which often lack the more common classical phenotype of PC. Although patient-derived xenograft studies can recapitulate PC subtypes, it is crucial to consider the inherent capacity of the tumor to engraft and grow when analyzing therapy response.

Our current measure of clinical trial grades drug response by RECIST criteria which does not take the complete patient health into account and focuses on reduction in tumor size alone. This overshadows many positive or negative effects a drug could be making on patient health. FDA released guidelines suggest incorporation of patient-reported outcomes or QOL(Quality of Life) assessments as primary, secondary, or

exploratory endpoints in clinical trials to develop therapeutic agents (USFDA 2009). A systematic review evaluated FDA approved cancer drugs and identified seven Trials that used validated health care related QOL assessment in inclusion criteria. Only one drug out of the seven retained FDA approval at the time of the study rest of the drugs produced either no effect, or worse, or mixed effects on QOL. European Medical Agency published similar guidelines encouraging QOL inclusion during the development of anticancer agents. Unfortunately, there are still limited studies using QOL endpoints in the registration of trials [87].

Lack of patient accrual and a need for evidence-based therapies warrants evaluation in trial designs and evidence generation. Basket trials that test drugs in different cancers that share common alterations could overcome to the lack of accrual. Umbrella trials testing different drugs targeting different alterations in a single tumor type could help generate more reliable evidence and better assessment of the clinical studies.

### 1.9 Conclusions

Pancreatic cancer is a very deadly malignancy with many hurdles to the development of effective therapy. There have been improvements in the last decade in our ability to address PDAC primarily due to better perioperative care and more efficacious adjuvant and neoadjuvant treatments. The survival following tumor resection in early stage PDACs has almost doubled to reach 30%. Due to potent neoadjuvant therapies, patients eligible for surgical resection have increased by nearly 50%. Although improvement in metastatic disease is still small new combination regimens, have been beneficial to patients. The overall survival for PDAC is still dismal, and better therapeutic interventions are needed. Even though Immunotherapies have still to yield a breakthrough for PDAC and approaches targeting stroma are still engulfed in

complexities, we have made a lot of progress in our understanding of PDAC. The elaborate molecular makeup and unique stroma leading to tumor heterogeneity, disease recalcitrance to therapies are much more understood. Development of new experimental models like organoid culture and technological advances in landscaping the cancer genome, precision medicine seems closer than ever. We need better analysis of results from our studies and reevaluate our study designs to incorporate the new information generated in recent years and including QOL as an endpoint in our studies to make sure that our therapies stand the test of time.

## 1.10. Figures and Legends

**Fig. 1.1 Treatment guidelines for potentially curable pancreatic cancer.**

Pancreatic cancer patients with borderline resectable or locally advanced disease are stratified according to performance status (defined by Eastern Cooperative Oncology Group score). The treatment chart represents a review of literature and guidelines from the American Society of Clinical Oncology.

Surgical removal of the primary tumor and regional lymph nodes is usually recommended for patients with no evidence of metastasis who meet the comorbidity profile. Neoadjuvant therapy can also be suggested to these patients but is usually suggested when there are radiographic findings suspicion of metastasis, but no diagnostic evidence and comorbidity profile does not allow major surgery.

Patients that undergo surgical resection with no preoperative therapy are suggested 6 months of adjuvant treatment, which is initiated within 8 weeks of surgery, assuming complete recovery and absence of medical or surgical contraindications. A doublet treatment with capecitabine and gemcitabine is preferred in absence of toxicity concerns. Alternatively, patients can be offered monotherapy with gemcitabine or fluorouracil plus folinic acid. Adjuvant chemoradiation may be suggested to patients (no preoperative therapy) with microscopically positive margin (R1) and/or metastasis in lymph nodes after 4 to 6 months of systemic adjuvant chemotherapy.

Patients who receive neoadjuvant treatment 6 months of total adjuvant therapy (including preoperative regimen) is recommended as per doctors' decision.

**Fig. 1.1**

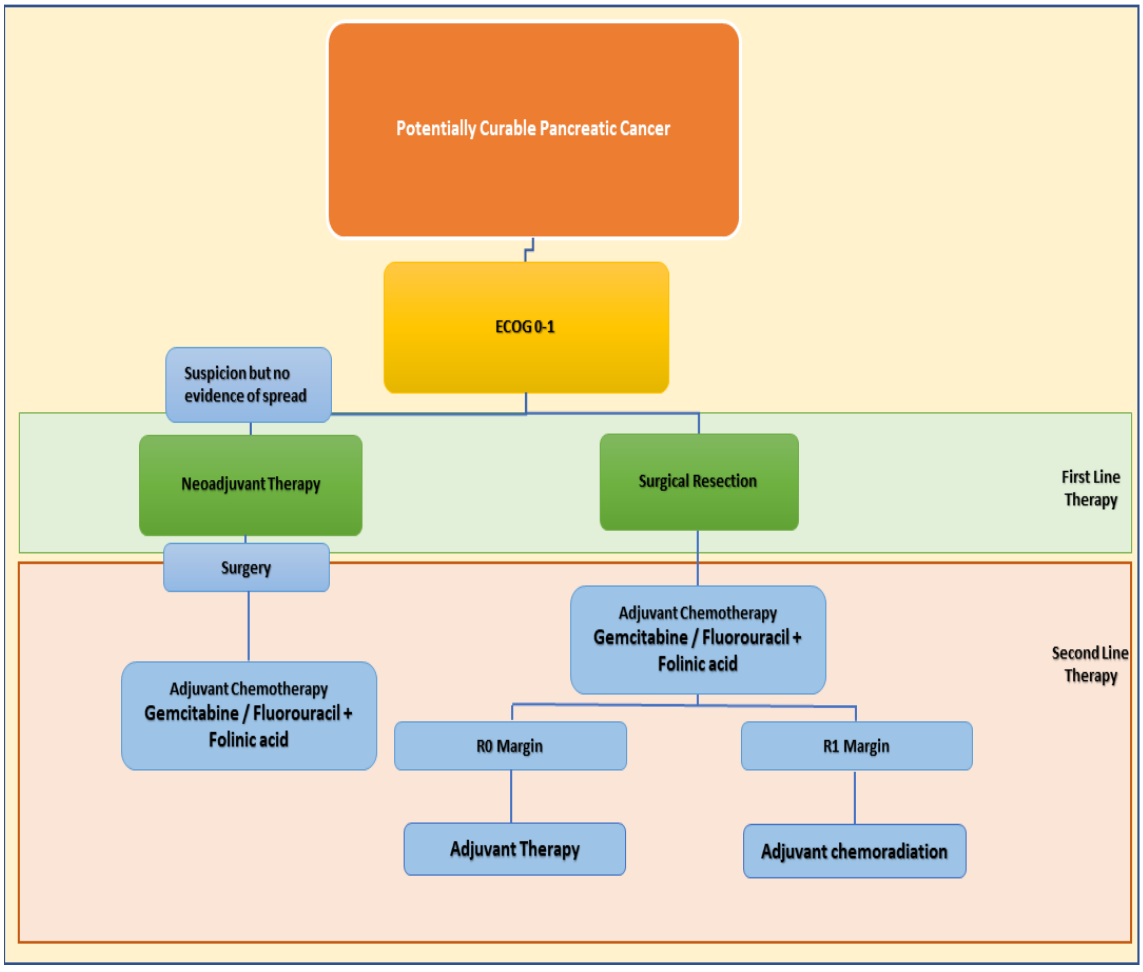

Potentially Curable Pancreatic Cancer
ECOG 0-1
Suspicion but no evidence of spread
Neoadjuvant Therapy
Surgical Resection
First Line Therapy
Surgery
Adjuvant Chemotherapy Gemcitabine / Fluorouracil + Folinic acid
Adjuvant Chemotherapy Gemcitabine / Fluorouracil + Folinic acid
Second Line Therapy
R0 Margin
R1 Margin
Adjuvant Therapy
Adjuvant chemoradiation

**Fig. 1.2 Treatment guidelines for borderline resectable or locally advanced pancreatic cancer**.

Pancreatic cancer patients with borderline resectable or locally advanced disease are stratified according to performance status (defined by Eastern Cooperative Oncology Group score). The treatment chart represents a review of literature and guidelines from the American Society of Clinical Oncology.

An induction chemotherapy (initial systemic therapy) is recommended for most patients with ECOG PS of 0 or 1 with favorable morbidity profile. Due to absence of randomized clinical trial data physicians may offer therapy on the basis of treatment of a metastatic PDAC. chemoradiotherapy or stereotactic body radiation therapy may be offered.

chemoradiotherapy or stereotactic body radiation therapy may also be suggested to patients in case of local disease progression after induction therapy but lack of evidence for systemic spread and ECOG PS less than equal to 2. patients who have stable disease or responded to 6 months of induction chemotherapy but developed unacceptable chemotherapy-related toxicities can also be given chemoradiotherapy or stereotactic body radiation.

Patients who do not benefit from first-line treatment should be offers treatment as per metastatic cancer guidelines.

**Fig. 1.2**

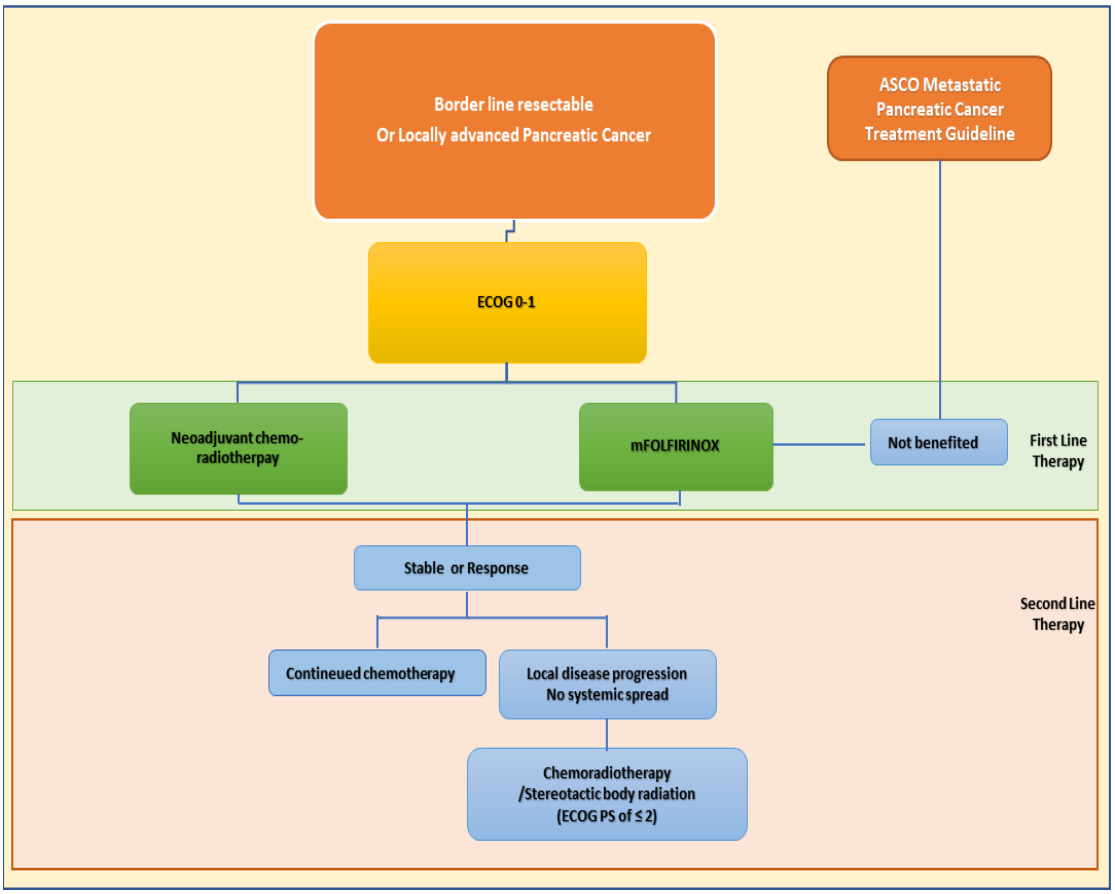

Border line resectable
Or Locally advanced Pancreatic Cancer
ASCO Metastatic
Pancreatic Cancer
Treatment Guideline
ECOG 0-1
Neoadjuvant chemo-
radiotherpay
mFOLFIRINOX
Not benefited
First Line
Therapy
Stable or Response
Second Line
Therapy
Contineued chemotherapy
Local disease progression
No systemic spread
Chemoradiotherapy
/Stereotactic body radiation
(ECOG PS of ≤ 2)

**Fig. 1.3 Treatment guidelines for metastatic pancreatic cancer.**

Pancreatic cancer patients with metastatic pancreatic are stratified according to performance status (defined by Eastern Cooperative Oncology Group score). The treatment chart represents a review of literature and guidelines from the American Society of Clinical Oncology.

**First-Line Treatment**

For patients with ECOG PS of 0 to 1 and favorable comorbidity profile FOLFIRINOX (leucovorin, fluorouracil, irinotecan, and oxaliplatin) is recommended when there is access to infusion pump. Gemcitabine plus NAB-paclitaxel is recommended for patients with an ECOG PS of 0 to 1 and a relatively favorable comorbidity profile.

Whereas for patients who have an ECOG PS of 2 and/or a comorbidity profile that disqualifies more aggressive regimens monotherapy with gemcitabine is recommended which may be supplemented with either capecitabine or erlotinib as per the doctor's decision.

Patients with an ECOG PS ≥ 3 or with poorly controlled comorbidity profile should be offered cancer-directed therapy only on a case-by-case basis with emphasis on optimizing supportive care.

**Second-Line Treatment**

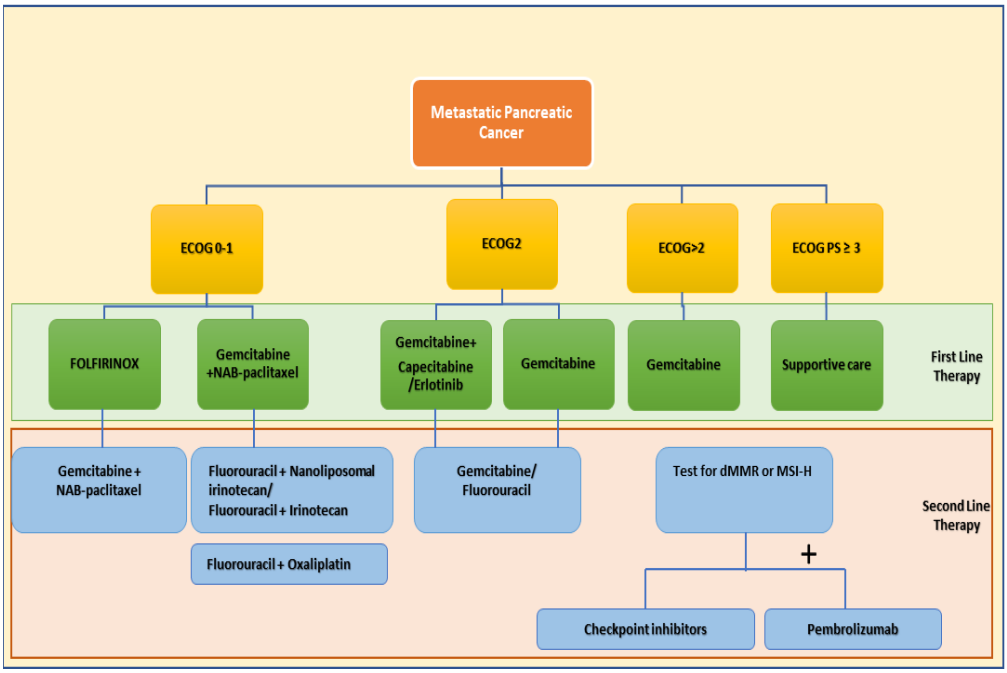
Routine testing for dMMR or MSI-H is recommended, for patients for checkpoint inhibitor therapy consideration. If tested positive a second line therapy with pembrolizumab may be suggested.

FOLFIRINOX treated patients can be given Gemcitabine plus NAB-paclitaxel as second-line therapy if patients maintain an ECOG PS of 0-1, a relatively favorable comorbidity profile and can sustain aggressive medical therapy. Fluorouracil plus nanoliposomal irinotecan, or irinotecan can also be suggested for such patients.

gemcitabine plus NAB-paclitaxel treated patients can be advised Fluorouracil plus oxaliplatin as second-line therapy if patients can support aggressive therapy and have an ECOG PS of 0 to 1, and relatively favorable comorbidity profile.

A second-line therapy of Gemcitabine or fluorouracil can be considered if patients have an ECOG PS of 2 or a comorbidity profile that disqualifies more aggressive regimens.

**Fig. 1.3**


Metastatic Pancreatic Cancer
ECOG 0-1
ECOG2
ECOG>2
ECOG PS ≥ 3
FOLFIRINOX
Gemcitabine +NAB-paclitaxel
Gemcitabine+ Capecitabine /Erlotinib
Gemcitabine
Gemcitabine
Supportive care
First Line Therapy
Gemcitabine + NAB-paclitaxel
Fluorouracil + Nanoliposomal irinotecan/ Fluorouracil + Irinotecan
Fluorouracil + Oxaliplatin
Gemcitabine/ Fluorouracil
Test for dMMR or MSI-H
+
Checkpoint inhibitors
Pembrolizumab
Second Line Therapy

# CHAPTER 2: MATERIALS AND METHODS

### 2.1 Cell culture

Capan1 and SW1990 cell lines were obtained from ATCC and were cultured in high glucose DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. Cultures were routinely inspected for mycoplasma contamination and phenotypic variation. Cell lines were incubated in a humidified atmosphere at 37°C with 5% $CO_2$. Afatinib was purchased from Selleck Chemicals, TX, USA, while gemcitabine was obtained from Sigma Aldrich. Afatinib and gemcitabine were reconstituted in PBS as per manufacturer's instructions.

### 2.2 Isolation of CSC/side population

The side population/CSC population was sorted using BD FACS Aria (BD Biosciences) after staining cells as shown previously [88]. One million cells in 1 mL of culture medium were stained with Hoechst 33342 (AnaSpec Inc., Fremont, CA, USA) (5 µg/ml) for one h at $37^0$C. Verapamil (Sigma) control staining was performed at a final concentration of 50 µM to set up side population gating.

### 2.3 Tumorsphere assay and drug treatments

An *in vitro* tumorsphere assay was performed using CSC and non-CSC populations isolated from SW1990 and Capan1 cells. The tumorsphere assay was performed as shown previously [89]. Briefly, cells were subjected to drug treatments and then seeded in low-attachment 24-well plates in DMEM/F12 (Invitrogen, Grand Island, NY, USA) medium supplemented with 1% B27 supplement, epidermal growth factor (EGF) (20 ng/mL), and basic fibroblast growth factor (bFGF) (10 ng/mL). After seven days, spheres were viewed under the microscope, counted and photographed.

### 2.4 RNA isolation and quantitative real-time PCR (qRTPCR)

For quantitative mRNA analysis, RNA was isolated using the QIAGEN RNeasy mini kit (Qiagen, Valencia, CA, U.S.A.), and its concentration was determined using a NanoDrop ND 1000 Spectrophotometer. cDNA was synthesized using oligo(dT)18 primer, and SuperScript II RNase reverse transcriptase (Invitrogen). qRTPCR was performed for genes that were screened by PCR array analysis.

### 2.5 Transfection experiments

SW1990-SP PC cells ($0.5\times10^6$) were seeded in a 6-well plate and incubated for 24 h. After 24 h, cells were transiently transfected with two independent siRNA oligonucleotides specific for human EGFR (100 pmol) (Origene) and FOX2 (100 pmol) (Origene) using lipofectamine transfection reagent for 72 h. Non-targeting (siRNAs) oligonucleotides were used as transfection control. After 72 h of incubation, cell lysates were isolated using RIPA buffer and subjected to western blotting and protein expression analysis.

### 2.6 PC organoid development from LSL-$Kras^{G12D/+}$; Pdx-1-Cre (KC), and LSL-$Kras^{G12D/+}$; LSL-$Trp53^{R172H/+}$;Pdx-1-Cre (KPC) mouse tumors

In brief, tumor organoids were established from KC and KPC autochthonous mouse models and subjected to enzymatic digestion with 0.012% (w/v) collagenase XI (Sigma) and 0.012% (w/v) dispase (GIBCO) in DMEM media containing 1% FBS (GIBCO); the extracts were embedded in growth factor-reduced Matrigel (BD Biosciences) [90]. Organoids were maintained and cultured in complete AdDMEM/F12 medium supplemented with HEPES (Invitrogen), Glutamax (Invitrogen), penicillin/streptomycin (Invitrogen), B27, Primocin (1 mg/ml, InvivoGen), N-acetyl-L-cysteine (1 mM, Sigma

Aldrich), mouse recombinant Wnt3a (100 ng/ml, EMD Millipore), human recombinant RSpondin1 (1 µg/ml, PeproTech), Noggin (0.1 mg/ml, PeproTech), epidermal growth factor (EGF, 50 ng/ml, PeproTech), gastrin (10 nM, Sigma), fibroblast growth factor 10 (FGF10, 100 ng/ml, PeproTech), Nicotinamide (10 mM, Sigma), and A83-01 (0.5 mM, Tocris Biosciences).

### 2.7 In *vivo* xenograft mouse model and treatment strategy

Luciferase-labeled Capan1 cells ($0.25x10^6$ cells) were injected *in vivo*, orthotopically in the head of the pancreas in athymic nude mice. The successful injection was verified by the appearance of a fluid bubble without signs of intraperitoneal (IP) leakage. The abdominal wall was sutured, and the skin was closed with wound clips. Following tumor development in these mice (4 weeks after injection), we started therapy. Afatinib was administered orally (15 mg/kg/body weight) 45 times a week, and gemcitabine was given IP (50 mg/kg/body weight) twice a week. Following three weeks of treatments, mice were euthanized, and primary tumors and other organs were harvested for further analysis.

### 2.8 Immunoblot analysis

Western blot analysis was performed as described previously [91]. The blots were incubated with the following primary antibodies: pEGFR, EGFR, pHer2, Her2, pERK, ERK, FOXA2, SOX9, CD44V6, CD44, EpCAM, ALDH1, ABCG2, pFAK, FAK, NANOG. Secondary antibodies used were Rabbit, 1:1000; Cell Signaling Technology (CST)) and β-Actin (mouse, 1:5000; Sigma Aldrich).

### 2.9 Confocal immunofluorescence microscopy

Immunofluorescent labeling of cells was carried out by plating them at low density on sterilized coverslips. Cells were washed with 0.1mol/L HEPES containing Hank's buffer solution followed by fixation with ice-cold methanol at -20°C for 2 minutes. Blocking was performed with 10% goat serum for at least an hour to avoid nonspecific staining. Primary antibody incubation was performed overnight at 4°C followed by PBS washes. The fluorescent conjugated secondary antibody was incubated for 1 hour at room temperature in the dark. The coverslips were inverted and mounted with Vectashield DAPI. Microscopic slides were prepared from formalin-fixed, paraffin-embedded samples of tumoroids (tumor organoids) and orthotopic tumors, de-paraffinized using xylene, and hydrated by a graded series of ethanol washes. Antigen retrieval was performed by microwave heating in 10 mmol/L sodium citrate buffer (pH 6.0) for 10 min. Sections were then probed with primary antibodies (1:200) diluted in PBS and incubated overnight at 4°C. After washing twice with PBS, secondary antibodies (1:250) were added for one hour. Slides were again washed twice with PBS and images were taken under a confocal microscope.

### 2.10 Immunohistochemistry

Microscopic slides were prepared from formalin-fixed, paraffin-embedded samples of orthotopic tumors, de-paraffinized using xylene, and hydrated by a graded series of ethanol washes. Antigen retrieval was performed by microwave heating of slides in 10 mmol/L sodium citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase activity was quenched with 3% H2O2 in PBS for 30 minutes. After washing slides were subjected to 30 minutes of blocking with Vectastain normal horse serum to block nonspecific binding. Sections were then probed with primary antibodies (1:200) diluted in PBS and incubated

overnight at 4°C. Sections were then washed and incubated with horseradish peroxidase-labeled secondary antibody for 30 minutes. Slides were washed and incubated with ABC solution (Vector Laboratories). The final reaction with 3,3'-diaminobenzidine reagent (DAB) was carried as per manufacturers instructions to develop color. He slides were subsequently washed with water and counterstained with hematoxylin. This was followed by the dehydration process using increasing alcohol gradients and mounted with Vectamount permount mounting media(Vector Laboratories).

**2.11 Statistical analysis**

Statistical significance was assessed using two-tailed t-test and ANOVA tests using GRAPH PAD Prism software. All experiments were carried out in triplicates. For composite scoring of tissue array percentage of a cell stained (0-100%) were multiplied with the intensity of the stain (1-3) to achieve a composite score. Differences between the groups were considered significant when the p-value was less than 0.05.

# CHAPTER 3: THREE-DIMENSIONAL ORGANOIDS AS PRECLINICAL MODELS

A part of this chapter has been published in as a review titled: **Concise Review: Current Status of Three-Dimensional Organoids as Preclinical Models [92]**

## 3A. Review of Literature

### 3A.1 Introduction

The past decade has seen tremendous development in disease modeling and generating accurate experimental models that mimic biological processes, from co-culture techniques to 3D printed scaffolds and organoid culture [93-97]. Generating precise experimental models is essential for understanding basic biology, disease development, and therapy responses, but generating the complex biological environment of an organ to study development, or tumor to study progression and therapy, is nevertheless a challenging task.

Over time, various tools have been employed to generate experimental models that can recapitulate human biology (or at least some of its properties). Conventional cultures have included growing transformed cells derived from biological tissues in monolayer cultures. These are easy to culture and amenable to experimental modifications. Though these transformed cell lines allow study of human cancer cells, because they have spent years *in vitro* since establishment, they tend to acquire an undefined and complex mutational background [98]. Monolayer cultures are also two-dimensional (2D) and do not represent the tissue architecture and cellular heterogeneity found in tissues or tumors of their origin [98, 99].

Animal models make for some of the drawbacks of 2D cultures since they include stroma, vasculature, and immune components. They can, however, be engineered to generate transgenic disease models to recapitulate pathogenesis using molecular biology tools and breeding strategies. Another experimental use of these animals is generating patient-derived xenografts or tumor xenografts. These models are extremely powerful but are also resource intensive and time-consuming to develop. Moreover, the genomic profile of animal models does not exactly march with human profiles [100].

The first reports that described 3D culture systems discussed models that allow for long-term expansion of single mouse adult intestine [101], stomach [93], liver [102], and pancreas [102]. The first breakthrough experiments in the field of 3D culture were performed using Lgr5+ stem cells or intestinal crypt cells in 2009 [101]; this study demonstrated that stem cells could be used to generate stable, near-physiological epithelia when supplied with growth factors and proteins close to endogenous stem cell niche components [101].

The idea of a system that recapitulates a holistic microenvironment of normal biology *ex vivo* while proving experimental ease and feasibility of cell lines lead to the development of 3D culture methods. Cellular behaviors *in vivo* depend on environmental signals and contacts with neighboring cells and the extracellular matrix. 3D cultures allow for these signals to some extent and hence serve as an experimental system closer to normal biology.

Organoid 3D-cultures can be formed using a variety of source materials, from spheroids derived from sorted cells to tissue segments, to whole organ explants [96, 97, 101, 103]. Due to the differences in source material used and the 3D environment and scaffold provided, there are differences in the types of cultures generated, and it is this diversity that poses a challenge to define these organoids. Although the definition of organoids is still evolving, for the purposes of this review, we use the term to address cultures that recapitulate *in vivo* architecture, maintain stem cells or progenitor pool, and multi-lineage heterogeneity.

Several approaches used to study a broad range of developmental and cellular processes have been comprehensively covered in reviews elsewhere [103, 104]. Our focus herein is on the most recent developments in organoid culture for major organs and cancers, including representation of their microenvironments and stem cells (niches)

on an appropriate scaffold. We also present our views on their implications on the development and testing of therapeutics.

### 3A.2 Perquisites to Organoid Generation: Where do we start?

Organoids have become a powerful tool for research and are becoming common in everyday lab practice, but there are certain key pieces of information necessary to consider before organoid development. Paramount among these are the selection of appropriate sources of organ or cells (iPSCs, Adult SCs, Organ chunks, embryonic SCs, etc.) and appropriate protocols that employ necessary growth factors and morphogens. The correct matrices essential for multiple stages of organoid development must also be determined. Then, once organoids develop, media composition must be determined to maintain structure and retain the functional characteristics of the organ of intent. In the following sections, we discuss such considerations and how they affect the generation of organoids.

### 3A.3 Source Tissue: Beginning decides the end

Organoids can be cultured from embryonic stem cells (ESCs), adult stem cells (AdSCs) , human-induced pluripotent stem cells (hPSCs), and tissue fragments. Development of hPSCs and human embryonic stem cells (ESC) culture techniques in parallel to 3D culture systems has helped researchers recapitulate the successful differentiation and development of endodermal (lung, stomach, liver, small intestine) and ectodermal (brain and retina) tissues *in vitro,* and has opened up new avenues for further research (**Fig. 1**).

Recent attention has focused on using hPSCs or patient tissue samples via the process of reprogramming adult somatic cells into induced pluripotent stem cells (iPSCs) by ectopic expression of pluripotency transcription factors [95]. These cells are then

transformed into organoids by using the signaling pathways involved in modeling germ layer formation and induction of organ primordia,Wnt, EGF, Retinoic acid, and TGFβ/BMP. iPSC-derived organoids have been generated from brain [105], lung [95], intestine [106], stomach [107], eyes [108], and kidney [109]. Organoids have also been generated using adult stem cells or adult primary tissue and then expanded long-term *in vitro*. These studies also built upon the available knowledge of stem cell niche requirements and generated their media with a base composition of Wnt, RSPONDIN, EGF, and Noggin, and include mouse and human pancreas, liver, intestine, stomach, prostate, fallopian tube, and salivary gland organoids [110]. Such availability of different sources determines the necessary media components.

### 3A.4 Importance of Matrix Selection

Once selected, it is important to provide an appropriate microenvironment for source tissue. The most common 3D environment used in the formation of organoids are hydrogels, such as Matrigel, that contain gelatinous mixtures of extracellular matrix components, including laminin and collagen [111, 112]. For instance, a primary culture of pancreatic ductal cells has been made possible by seeding stem cells or tissue fragments in Matrigel as submerged cultures that support the growth of epithelial cell cultures [93], whereas long-term organoid cultures that include both epithelial and mesenchymal components have been successfully performed using an air-liquid interface method [93, 100]. This method uses collagen gel containing trans-well suspended in media and direct exposure to air. Tissues or cells are mixed with Matrigel and layered over the collagen, where they develop organoids. The first study to engage this method developed a primary mouse intestinal culture that allowed for sustained

intestinal proliferation and multi-lineage differentiation. Such cultures lasted from 30 to >350 days using neonatal tissue as starting material [113]. This allowed recapitulation of both the cellular myofibroblast architecture and the rigorous Wnt and Notch-dependent stem cell compartment. These organoids could then be transformed into their oncogenic counterparts by genetic manipulation [114].

A recent study reported that matrix stiffness has an impact on organoid growth and stem cell signaling in intestinal organoids. The authors reported employing a synthetic scaffold design, using a polyethylene glycol backbone with a more consistent and chemically defined synthetic hydrogel that allowed stem cell expansion and organoid formation [115]. Similarly, artificial scaffolds can be created by attaching microenvironmental signals such as ECM components and cell-to-cell interaction proteins to an artificial scaffold [115]. Various techniques may be tried, such as microcontact printing, which directly deposits ECM onto a partially polymerized hydrogel substrate using soft lithography techniques [116]. Nutrition and gaseous exchange are two other aspects affected by the matrix. Human bronchial epithelial cells(HBECs) embedded within Matrigel, for example, organize into spheroids that contain cuboidal epithelial cells, whereas HBECs plated on top of the Matrigel layer self-organize into tubular structures that undergo branching and budding.

### 3A.5 Media requirements and properties of generated Organoids

Developmental biology studies have long revealed that the morphogen gradient decides cell fate during embryo development. 3D organoids have been used to intelligently apply that knowledge when deciding on which media components will be necessary for a specific organoid generation. We will further describe media requirements and the

rationale behind several organoids that have been developed and used for modeling disease.

***Brain Organoids***

3D brain tissues or mini brains can now be generated using pluripotent stem cells (PSC). These tissues, also called cerebral organoids, are created by first driving pluripotent stem cells to a neural progenitor lineage and then providing a supportive 3-dimensional microenvironment for them, where they can self-organize spontaneously into the early embryonic brain [117]. A recent study reported the successful formation of brain organoids from human pluripotent stem cells, comprising the timely amalgamation of several previously published methodologies to accomplish successful differentiation of PSC into neural progenitors and progress further down the neural lineage [105, 118].

Mototsugu Eiraku reported in 2008 a special 3 D culture condition that showed differentiation of embryonic stem cells into embryoid bodies and then into self-organized apicobasal polarized cortical tissue [119]. He used FGF, Wnt, and BMP factors to model his 3D aggregate cultures. Since then, multiple reports have built upon his methodology [97, 105, 117, 118, 120-122]. A more recent study reported the development of heterogeneous brain organoids, naming them “cerebral organoids.” They used a similar approach and differentiated embryonic cells to neuroectoderm and then incubated them in differentiation media in Matrigel, finally allowing them to grow and form cerebral organoids in differentiation media supplemented with retinoic acid as Matrigel droplets in a spinning bioreactor [117].

A recent report described a novel developmental model of 3D brain-like tissue by applying an interdisciplinary approach, involving seeding cells within a biomaterial

scaffold to assemble microstructural features representative of native tissue [118]. This technique sought to recapitulate the structural features formed during development of the forebrain cerebral cortex, including gray matter (containing cell bodies) and white matter (containing neuronal axons). The study used silk protein to design scaffold, which provided spatial separation of cell bodies and neural processes. This lead to the development of a suitable matrix for growth of 3D compartmentalized neuronal networks that could recapitulate the properties of the native cortex and to establish the suitable conditions for neural growth [105, 122].

***Lung Organoids***

During embryonic development, the endoderm produces a primitive gut tube along which the lung, thyroid, and organs lining the gastrointestinal (GI) tract emerge. The lung arises from cells expressing the transcription factor NKX2.1 (TTF-1; Thyroid Transcription Factor 1) in the ventral wall of the anterior foregut endoderm [123]. Hence, current available protocols include discrete steps to differentiate human pluripotent stem cells (hPSCs) through an initial definitive endoderm (DE) specification, then the anterior foregut endoderm (AFE), and finally into ventral anterior foregut endoderm (VAFE) and NKX2.1 expressing lung progenitors [123, 124]. Each step uses stage-specific growth factors to recapitulate the signaling pathways involved in lung development. Of importance, since organoids develop from tissue-specific stem cells or progenitors, hPSC differentiation into these cell types has been employed as a strategy to develop organoids.

Lung organoids are mostly derived from primary respiratory cells and cell lines [123, 125]. A recent study demonstrated that primary basal cells harvested from mouse and human lungs could self-organize into organoids, called tracheospheres or bronchospheres, when cultured in a 3D ALI [125]. These organoids are derived from

basal cells expressing p63 and NGFR, which proliferate to establish a layer of basal cells in a spherical organization, and that are covered on the luminal side by a second layer of a differentiated goblet and ciliated cells.

Thus far, two studies have reported the generation of lung organoids *in vitro* from hPSCs. The first showed that purified Carboxypeptidase M (CPM) expressing cells in 3D conditions, supplemented with alveolar-related growth factors and human lung fibroblasts, produced alveolar epithelial spheroids. These spheroids contained cells expressing NKX2.1 and CPM, as well as differentiated cells that stained positive for AQP5 (Aquaporin 5) and SFPTC (Surfactant Protein C), markers of type 1 and 2 alveolar epithelial cells (AEC1s and AEC2s), respectively [126, 127].

The second study performed a step-by-step differentiation of hPSCs and reported multi-lineage organoids with epithelial and mesenchymal components. By stimulating the Hedgehog (HH) signaling pathway during spheroid generation, the authors could enhance NKX2.1 expression and expand spheroids in media containing FGF10 [123, 126]. This allowed VAFE spheroids to grow into more complex structures that the authors called human lung organoids (HLOs). HLOs persisted in culture for over 100 days and developed organized proximal airway-like epithelial tubules containing numerous cell types found in the native airway epithelium, including basal, ciliated, and club cells and that were surrounded by smooth muscle actin (SMA)- expressing mesenchymal tissue maintaining early bi-potent alveolar progenitor cells [123, 126, 127]. In our lab, we explored these principles and generated lung organoids from genetically engineered mouse model with mutant KRAS and p53 deletion. We used a media composition that favors maintenance of stem cells and lung development by including factors such as FGF4, Noggin, and CHIR99021 (Glycogen Synthase Kinase 3 Inhibitor). These organoids generate histological structures similar to lung tumor and proved to grow stably in culture.

### *Mammary Organoids*

Mammary acini contain extensive stromal and extracellular matrix compartments, the composition of which changes depending on signals such as growth factors and hormonal changes. Even monolayer cultures of mammary epithelial cells can form functional tubular structures when provided with the required environmental cues; for example, upon transplantation into the gland-free fat pads of mice [128]. It would be logical to infer that either the systemic factors or cellular microenvironment that surrounds the mammary epithelial cells confer the cues that drive functional differentiation of mammary epithelial tissue, suggesting the importance of providing a matrix to the cells in culture that resembles their biological ECM. Michalopoulos and Pitot (1976) were the first to use floating collagen gels to provide an extracellular environment to hepatocytes that could maintain their functional and morphological identities in culture for a short period [129]. Emerman and Pitelka further adapted this idea in the 1970s, and in 2013, Mroue and Bissell cultured mouse primary mammary epithelial cells that retained functional differentiation using both floating collagen-I (Col-I) gels and laminin-rich ECM gels (lrECM) [128, 130]. Mammary epithelial cells grown on floating collagen gels were found to reorganize and form secretory structures that express milk proteins *de novo*.

Another observation made by Mroue et al. highlighted the significance of the composition of ECM provided in deciding the fate of cells in culture [130]. They reported that mammary organoids, when cultured on floating collagen gels, contained mammary epithelial cell (MECs) clusters, which exhibit basoapical polarity and cellular junctions,

and expressed the milk protein β-casein. These lacked expression of whey acidic protein (WAP) and did not form luminal alveolar structures, both essential features of the mammary gland [130].

In a recent report, Linnemann et al. published alternative 3D culture conditions for the expansion of TDLU-like structures (Terminal Ductal lobular Units) from primary human cells [131]. The advantage of this method is that it includes conditions that support the growth of single cells at high efficiency. Its drawbacks are that it incorporates chemical agents (Rho-associated protein kinase inhibitor, forskolin) and serum that perturb intracellular signaling in nonphysiological ways. The relative merits and failings must be carefully considered before deciding the appropriate model system to use in the future study.

***Liver Organoids***

Two epithelial cell types, hepatocytes, and ductal cells, chiefly compose liver [132]. Hepatocyte-like cells have been generated by differentiating human embryonic stem (hES) cells and human induced pluripotent stem (hiPS) cells. However, because of the genetic and epigenetic aberrations that occur during the reprogramming process [133, 134], the use of these models for translational research and regenerative medicine [135] remains limited. Generation of liver organoids overcomes these limitations; the organoids are generated by using the Wnt, BMP, RA, HGF, and FGF signaling pathways that regulate the embryonic development of the liver.

Liver organoids were generated by mixing stem cells and/or tissue fragments with Matrigel and providing growth factors like EGF, HGF, FGF, and RSPO1. Such conditions allowed liver cells to self-organize into organoids resembling embryonic liver buds [136]. These organoids were Keratin positive and expressed progenitor cell

markers. The authors also reported that the generation of human liver organoids requires inhibition of TGFβ signaling. Replacement of Notch and RSPO by dexamethasone and BMP also allowed differentiation of these organoids into hepatocytes [136].

***Pancreatic Organoids***

Two functionally distinct compartments make up the pancreas: ductal and acinar cells consist of the exocrine compartment, and the Islets of Langerhans make up the endocrine compartment. The genes and molecular pathways regulating the embryonic development of the two compartments are evolutionarily conserved and include FGF, HGF, Wnt, BMP, RA, and TGFβ. These pathways promote survival, proliferation, and migration of the progenitor pools that generate these two distinct compartments [93, 101, 102, 137, 138]. Developmental studies and knowledge acquired from 3D cultures of the stomach and intestine allowed researchers to culture, expand, and differentiate mouse and human primary pancreatic tissue [101-103]. Providing an ECM and a microenvironment that includes growth factors essential during development of pancreas (Noggin, EGF, FGF and R-spondin-1(Rspo1)) is necessary for the long-term expansion of the adult pancreatic tissue in these 3D culture systems [102]. Using a similar approach, we developed murine pancreatic organoids from wild-type C57BL/6 murine pancreas which show histology similar to pancreatic ducts (Fig2.). A similar approach was also used to generate models to study pancreatic tumors and for which tumor organoids were generated from KC (*Kras;PdxCre)* and KPC (*Kras;p53;PdxCre*) autochthonous animal models for PDAC and were shown to histologically represent PDAC progression.

Another study used neonatal wild-type C57BL/6 mice pancreatic tissue to generate organoids by the air-liquid interface culture method. These grew progressively

for more than 30 days as cystic structures carrying an epithelial layer and surrounded by fibroblasts [139]. α-smooth muscle actin–positive ($SMA^+$) stromal cells were observed in association with these cystic epithelial organoids, which could be readily infected with adenovirus and were predominantly comprised of E-cadherin–positive ($E\text{-}cad^+$) and $Pdx1^+$ ductal epithelium with $PCNA^+$ proliferating cells. [93, 140]. Somatostatin and insulin were also found to be expressed in rare, islet-like regions not always associated with ductal structures; sporadic immunoreactivity was also reported for glucagon and amylase occasionally.

***Intestinal organoids***

A submerged Matrigel model [101] allowed a culture of $Lgr5^+$ cells or crypt fractions to be cultured into exclusively epithelial organotypic intestinal structures, also referred to as enteroids. Growth factors that simulate the paracrine signaling environment surrounding stem cells were supplemented in a media concoction for these enteroids, and 3D mouse and human organoids were generated [101]. Intestinal organoid or enteroid generation by this method requires Wnt stimulation with Wnt3A, epidermal growth factor (EGF), the BMP antagonist Noggin, and the Wnt agonist R-Spondin1 to be supplemented in the media [141]. Another recent method uses collagen-based, air-liquid interphase to generated 3D organoids which incorporate epithelial and mesenchymal components [142]. This system uses collagen and Matrigel together to grow organoids on air-liquid interphase, without the requirement of exogenous growth factors, and the resulting organoids were found to contain both epithelial and mesenchymal compartments and could be cultured for >60 days [143]. Primary intestinal and tumor organoids have been generated using both these methods and are currently being used for a variety of applications, from toxicology studies to drug screens and disease modeling [143].

Epithelial enteroids propagated from mouse or human $Lgr5^+$ cells or intestinal crypt fractions follow an easy-to-follow method of generation and expansion. Enteroids

typically constitute cystic epithelium with an inward-facing apical side and a progenitor pool containing crypt-like invagination [144]. All intestinal lineages have been observed in these cultures, which could be serially passaged and expanded. To enhance efficiency and include multiple biological parameters, several refinements have been made to this system, including Wnt3A withdrawal used to facilitate differentiation; the use of the Rho kinase inhibitor Y-27632 to avoid anoikis during tissue preparation, passaging and revival; using GSK3β kinase inhibitors and valproic acid to promote a stem-like state; and myofibroblast feeder layers to replace Wnt and R-spondin from medium [143]. Isolation of these enteroids follows a general scheme of mincing tissue or intestine and subjecting it to enzymatic digestion. The digested fraction is centrifuged, and intestinal crypts are isolated from it. These crypts are then embedded in Matrigel and seeded in submerged cultures [101].

***Ovarian and Fallopian Tube Organoids***

Fallopian tube model systems have traditionally facilitated ovarian development and ovarian cancer studies, and a similar trend was followed in 3D culture systems. Recent studies have demonstrated the existence of adult stem cells in the human fallopian tube epithelium that gives rise to differentiated epithelial cells in complex 3D organoids *in vitro* [145]. These organoids were reported to recapitulate the *in vivo* tissue architecture; they could depict growth and be maintained in culture long term. The culture protocol and growth conditions are similar on a gross scale to the intestinal tract, skin, liver, and ovary. Active Wnt signaling works towards maintaining the stem cell population in these organoids, achieved by activating Wnt target genes Lgr4, 5, and 6, all of which are a subfamily of leucine-rich, repeat-containing G protein-coupled receptors that can strongly amplify Wnt signals. The R-spondin family of proteins also acts as an Lgr receptor agonist. Similarly, the growth capacity of organoids is modulated by Wnt3A and R-spondin-1 (RSPO1) [145]. This organoid model faithfully mimics the normal physiology

and anatomy of the human fallopian tube and provides a platform for future investigations into the regulatory mechanisms involved in its cellular renewal and pathology. Overall growth rates for these organoids during long-term culture remained constant, with passaging every 2–3 weeks. This method yielded expandable, stable organoid cultures in all healthy tissue samples, with minimal variations in sphere formation potential and growth rate between donors or between distal and proximal tubal regions

***Prostate Organoids***

The prostate gland produces seminal fluid in the male reproductive system and is composed of a pseudostratified epithelium of luminal, basal and rare neuroendocrine cells. Additionally, prostate development and homeostasis, as well as prostate cancer initiation and progression, requires androgen receptor (AR) signaling [146]. Most *in vitro* studies are performed using cell lines, and most of these do not have an intact AR signaling pathway, making them poor models to represent healthy prostate and cancer tissue [146, 147]. Lack of suitable *in vitro* model systems is an obstacle for prostate cancer research. Although robust *in vivo* models are available, these are often expensive, time-consuming, and technically daunting.

Another study by Chua et al. recently demonstrated the development of organoids from sorted luminal cells, but these 3D cultures with limited growth potential [148]. Although these were AR-responsive, the medium used in this study included undefined components like fetal calf serum and had a plating efficiency of 0.2% to 0.3% [147, 148].

Drost et al. developed a testosterone-responsive prostate organoid culture system by adapting and optimizing the culture conditions previously employed to establish mouse and human colon and intestinal organoid cultures. Different compounds and growth factors, including epidermal growth factor (EGF), Noggin, and R-spondin 1,

were added to the generic organoid medium to allow for the establishment of long-term mouse and human prostate organoid cultures. These submerged prostate organoid culture systems contain multipotent progenitor cells in both the luminal and basal lineages that can be propagated long term. AR signaling is indispensable for organoids to functionally recapitulate prostate and was maintained in these organoids. This study concluded that organoids derived from human or mouse prostate cancer recapitulate genetically and phenotypically the tumor from which they were derived [147].

This method was further adapted by our lab to generate prostate and prostate cancer organoids from mouse tissues (**Fig. 2**). The main procedure involves dissection and digestion of tissue, followed by subsequent embedding, plating, and organoid passaging. We plated organoids are plated in Matrigel and cultured them in a defined prostate culture medium, after which they were sub-cultured and frozen. These organoids can be cryopreserved once established and are genetically and phenotypically stable. Prostate tumor organoid media includes DMEM/F12 supplemented with B27, N-acetylcysteine, EGF, Noggin, R-spondin 1, A83-01, and DHT [147, 149].

### 3A.6 Limitations: Need for Intervention

The potentials organoids hold for future study and use are innumerable, but they present some limitations, as does any technology. The organoids established so far need to be characterized and studied to the extent that exact recapitulation of *in vivo* development is possible. Tissue maturation is one of the limitations associated with retinal and cerebral organoids, where early events display intact organization, but the organized tissue fails to develop into a functionally mature organ. However, intestinal organoids

produce Lgr5+ stem cells, implying movement towards a mature intestine [101]. One solution to the maturation problem may be by growing a mature organ followed by transplantation. Another limitation of organoids is non-vascularization due to limitations in nutrient supply, which may be solved by spinning bioreactors that provide a better nutrient exchange. Vascularization may also be achieved by co-culturing with endothelial cells that can generate vascular-like networks. Some researchers have generated hybrid cultures with organoids to incorporate different cell types to generate more insightful models.[150]

Another limitation to organoid cultures is the limited presence of stromal components, including immune components, and this hinders organoid use in modeling inflammation and drug penetration studies [151]. Organoid cultures are also heterogeneous with no reliable means of synchronizing size shape and viability. This, unfortunately, leads to complications in data analysis and study design. Although all these limitations stand in the way of organoid applications, they can be overcome by a better understanding of ECM components and live cell imaging techniques that facilitate the analysis of the co-cultures or hybrid cultures of these organoids.

### 3A.7 Prospective application: Making the most of tremendous tool

Normal organoids derived from stem cells and specific organs can be used for their molecular impact on organogenesis study. Here we discuss different areas of biological science research that organoids have applications in. (**Fig. 3**).

**Organoid developed for tissue modeling**

Organoids hold an advantage over traditional techniques to solve unanswered questions in developmental biology because of the accessibility of model systems, especially for human models. For example, the unique division mode of neural stem cells has been studied using human brain organoids [105, 117, 151]. Similarly, the differences in tissue

morphogenesis and timing between humans and rodents have been studied using retinal organoids. Furthermore, organoids may be used to study processes that differ in model organisms and humans, such as GI tract development, and to model adult homeostasis. Specifically, the role played by the crypt niche in self-renewal and differentiation of stem cells has been studied using intestinal organoids. Regenerative events in adult organs, such as liver and stomach, have also been closely recapitulated by organoids derived from adult progenitors.

**Organoids developed for regenerative medicine**

Organoids also provide an alternative for cell and whole organ transplantation by providing autologous tissue. Organ transplant procedures with high demands and low success rates, such as renal transplants, may be improved using corresponding organoids. Successful transplantation of kidney organoids in adult mice has been already achieved by Taguchi et al., paving the way for a promising future for organoid transplantation [109, 152]. Stem cell therapies are being aided by retinal organoids in clinical trials to replace degenerating cell types. On similar lines, intestinal organoids are also under investigation to treat damaged and diseased colon.

**Organoids used for therapy response in cancer and other pathologies**

The failure rate of traditional models of therapy testing translating to patient treatment regimens has launched a hunt for more reliable and physiological models, such as patient-derived organoids for drug screenings and drug discovery studies. Yin et al. recently reported the use of primary intestinal organoids for modeling retroviral infection and antiviral therapy in an attempt to address the lack of potent antiviral therapies [153]. In another study, Ogawa et al. used patient-derived cholangiocyte organoids to correct the misfolded CFTR protein (cystic fibrosis transmembrane conductance regulator) [154].Using a similar strategy, Schwank et al. used CRISPER/Cas9 to repair the function

of CFTR in intestinal stem cell organoids. Such studies highlight the utility of organoids in disease modeling and therapeutic testing [155].

Additionally, Huang et al., in a recent study generated pancreatic ductal organoids and induced mutations in them to successfully model pancreatic ductal adenocarcinoma. They then used these organoids to test the therapeutic efficacy of a histone methyltransferase inhibitor, working along the idea of disease modeling and therapy [156]. Skardal et al. established 3D liver organoids to model colorectal cancer metastasis and were able to test therapies on metastatic disease [150]. Van de Wetering et al. established tumor organoid cultures from 20 colorectal carcinoma patients representing most genetic subtypes of colorectal cancer generating a living biobank [157]. In these and other examples, we can see that organoid technology provides an opportunity to bridge the gap between patient-derived cell lines and xenograft mouse models, generating a link between cancer, genetic, and patient trials to make possible better and personalized therapy designs. All these studies suggest that 3D organoids models are still developing but have immense potential for modeling various diseases and use in therapy design.

### 3A.8 Conclusions and Perspectives: Towards modeling accurate homeostasis and disease

Organoid cultures are accessible and physiologically relevant models to study biology. They can be derived from multiple sources, and they maintain stem cell or progenitor population. [93, 103, 140, 144, 158]. These models are robust in recapitulating *in vivo* tissue biology and have shown to be reliable in testing therapeutic response. They have the capacity to serve as a platform for translational research and high throughput

preclinical screenings. Organoid technology has worked successfully with current research methodologies and found its niche.

More development will be seen in 3D organoid systems that will compensate for the limitations the technology still suffers, however. Despite this, we hope to see the extensive use of organoids in many more avenues. Patient-derived organoids provide an opportunity to develop personalized treatment regimens for patients since biopsies can be an excellent source of disease site tissues and normal tissues for deep sequencing. This would, in turn, reveal causal mutations and phenotypic profile to generate therapeutic approaches tailored to each patient. Once organoids from patients are generated, they can be used to test efficacy or resistance to proposed regimens. Additionally, organoids generated from healthy tissues can be used to weed out toxicity and other undesired effects of the proposed therapy.

Organoid studies are being used for disease modeling for developmental disorders, cancer, degeneration, and other infectious diseases [142, 144, 150, 153, 155, 159-162]. This may also be achieved by introducing patient mutations in human PSCs, using genome-editing techniques, by generating organoids or inserting mutations directly into organoids. Organoids are also being employed for screening drugs, testing for efficacy and toxicity by modeling different degenerative conditions such as liver fibrosis or cystic kidney diseases where effective treatment regimens are required. If successful, this approach could lead to a reduction of the use of animal testing, which would be reserved only for studies requiring whole-organism readouts.

Organoids have found their place in everyday research and to date have significantly supplemented our knowledge and ability to model diseases. We have seen an exponential increase in their usage and application since the first introduction. Their reliability, robustness, and amenability for research have yielded enormous downstream applications, highlighting their role in recapitulating homeostasis and diseases. These

characteristics make organoids extremely exciting and promising technology that holds a promising future for therapeutics.

### 3A.9 Figures and legends

**Fig. 3.1 Representation of the organoids generated, and the media compositions required**: These hosts of organoids have been generated from different source materials, including iPSCs, adult stem cells, embryonic tissues or cells and adult tissue explants. Different media compositions are required for each type of source material used and the type of differentiation to be achieved (organ-specific), which is elaborated in detail in the text. Specifically, **cerebral organoids** need a stepwise incubation of pluripotent stem cells in neural induction media (DMEM-F12, N2 supplement, GlutaMAX supplement, MEM-NEAA, heparin) followed by cerebral induction media (DMEM-F12, Neurobasal medium, N2 supplement, insulin, GlutaMAX supplement, MEM-NEAA, penicillin-streptomycin, 2-mercaptoethanol, B27 supplement). **Mammary organoids** can be developed from tissue fragments using media composed of DMEM/F12, FBS, ITS Selenite media supplement, FGF2, FGF10 for mouse or EpiCult B medium supplemented with hydrocortisone, insulin, FGF10, HGF for humans. **Liver organoids** can be generated by mixing tissue fragments in DMEM/F12 media supplemented with FBS, EGF, RSPO1, FGF, HGF, Nicotinamide, and insulin. **Pancreatic organoids** need a media comprising of DMEM/F12, B27 supplement, Nicotinamide, Noggin, EGF, FGF, and RSPO1. Ovarian organoids are generated by seeding fallopian epithelial cells in Matrigel with media comprising AdDMEM/F12, Wnt3A, RSPO1, HEPES, GlutaMAX, B27, N2 Supplement, EGF, noggin, FGF10, Nicotinamide, Y-27632, and SB431542. **Prostate organoids** need a media containing DMEM/F12, B27 Supplement, N-acetylcysteine, EGF, Noggin, RSPO1, A83-01, and DHT. **Kidney organoids** need a media containing DMEM high glucose, FBS, NEAA, GlutaMAX, Heparin, APEL media, FGF9, SB431542, and CHIR99021. **Gut or intestinal organoids** need a media

composition of DMEM/F12, FBS, B27, EGF, RSPO1, Noggin and Wnt. Specific cultivation of stomach organoids needs media composition same as intestinal organoids with the addition of FGF. Lung organoids can be generated and grown in media containing DMEM/F12, FBS, B27, N2 Supplement, GlutaMAX, FGF4, Noggin, SB431542 and CHIR99021.

Abbreviation used are: Y-27632:ROCK inhibitor, SB431542:TGF-β R Kinase Inhibitor IV, ITS: Insulin Transferrin-Sodium, NEAA: Non Essential Amino Acid Culture Supplement, EGF: Epidermal Growth Factor, RSPO1: R-spondin-1, Wnt3A: Wingless-Type MMTV Integration Site Family Member 3A, T3: Triiodothyronine, FBS: Fetal Bovine Serum, FGF: Fibroblast Growth Factor, HGF: Hepatocyte Growth factor, DMEM/F12: Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 , DHT: Dihydrotestosterone , CHIR99021: glycogen synthase kinase 3 inhibitor.

**Fig. 3.1**

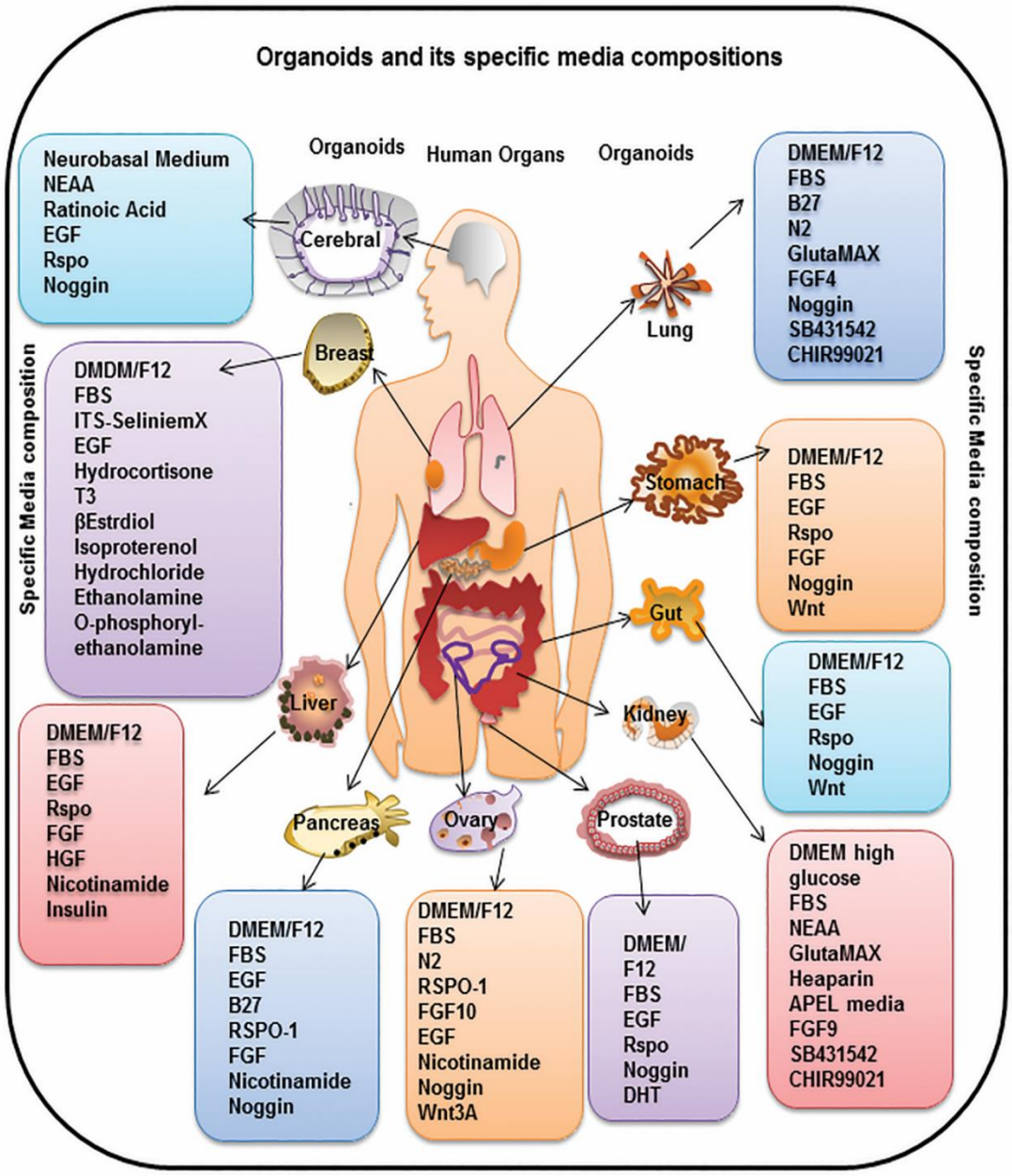

Organoids and its specific media compositions
Organoids
Human Organs
Organoids
Neurobasal Medium
NEAA
Ratinoic Acid
EGF
Rspo
Noggin
Cerebral
DMEM/F12
FBS
B27
N2
GlutaMAX
FGF4
Noggin
SB431542
CHIR99021
Lung
Specific Media composition
DMDM/F12
FBS
ITS-SeliniemX
EGF
Hydrocortisone
T3
βEstrdiol
Isoproterenol
Hydrochloride
Ethanolamine
O-phosphoryl-
ethanolamine
Breast
Stomach
DMEM/F12
FBS
EGF
Rspo
FGF
Noggin
Wnt
Gut
Liver
DMEM/F12
FBS
EGF
Rspo
Noggin
Wnt
Kidney
DMEM/F12
FBS
EGF
Rspo
FGF
HGF
Nicotinamide
Insulin
Pancreas
Ovary
Prostate
DMEM high
glucose
FBS
NEAA
GlutaMAX
Heaparin
APEL media
FGF9
SB431542
CHIR99021
DMEM/F12
FBS
EGF
B27
RSPO-1
FGF
Nicotinamide
Noggin
DMEM/F12
FBS
N2
RSPO-1
FGF10
EGF
Nicotinamide
Noggin
Wnt3A
DMEM/
F12
FBS
EGF
Rspo
Noggin
DHT

**Fig. 3.2 General scheme of generating organoids and representative figures of organoids generated by our laboratory:**

The flowchart represents the scheme of organoid isolation which is modified for each organoid according to the organ or tissue architecture to generate submerged organoids. Briefly, desired source tissue (progenitor cells or tissue fragments) is isolated from the host by mincing the organ and then subjecting it to enzymatic digestion. The digestion media composition and the digestion protocol are decided depending on the host tissue. The digestion media usually contains a mixture or Dispase and Collagenase or Collagenase alone and can take from 30 mins to 4-6 hours. Following digestion, the cells are mixed in the matrix (like Matrigel or collagen) suitable for the desired organoids. A suitable media is overlaid once the matrix solidifies. Once generated, organoids grow in ductal like morphologies like their human counterparts. Picture panels depict the microscopic pictures of organoids generated in our lab from normal and cancerous prostate and pancreas as well as lung cancer organoids (upper panel) along with hematoxylin and eosin stained sections of the same (lower panel) depicting the difference in the organization of cells in each of these organoids. Figure magnifications are mentioned in each of the figures.

**Fig. 3.2**

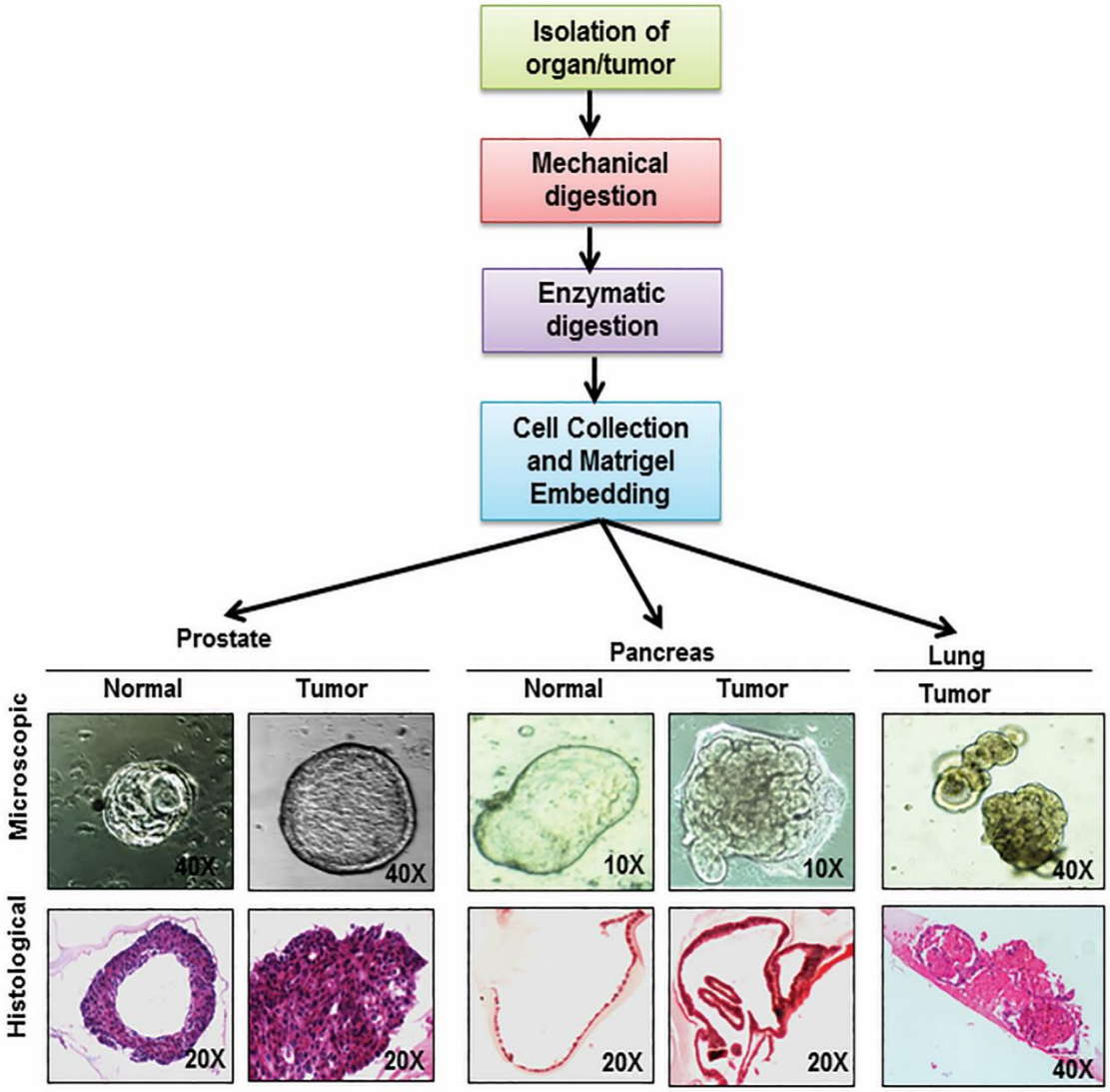
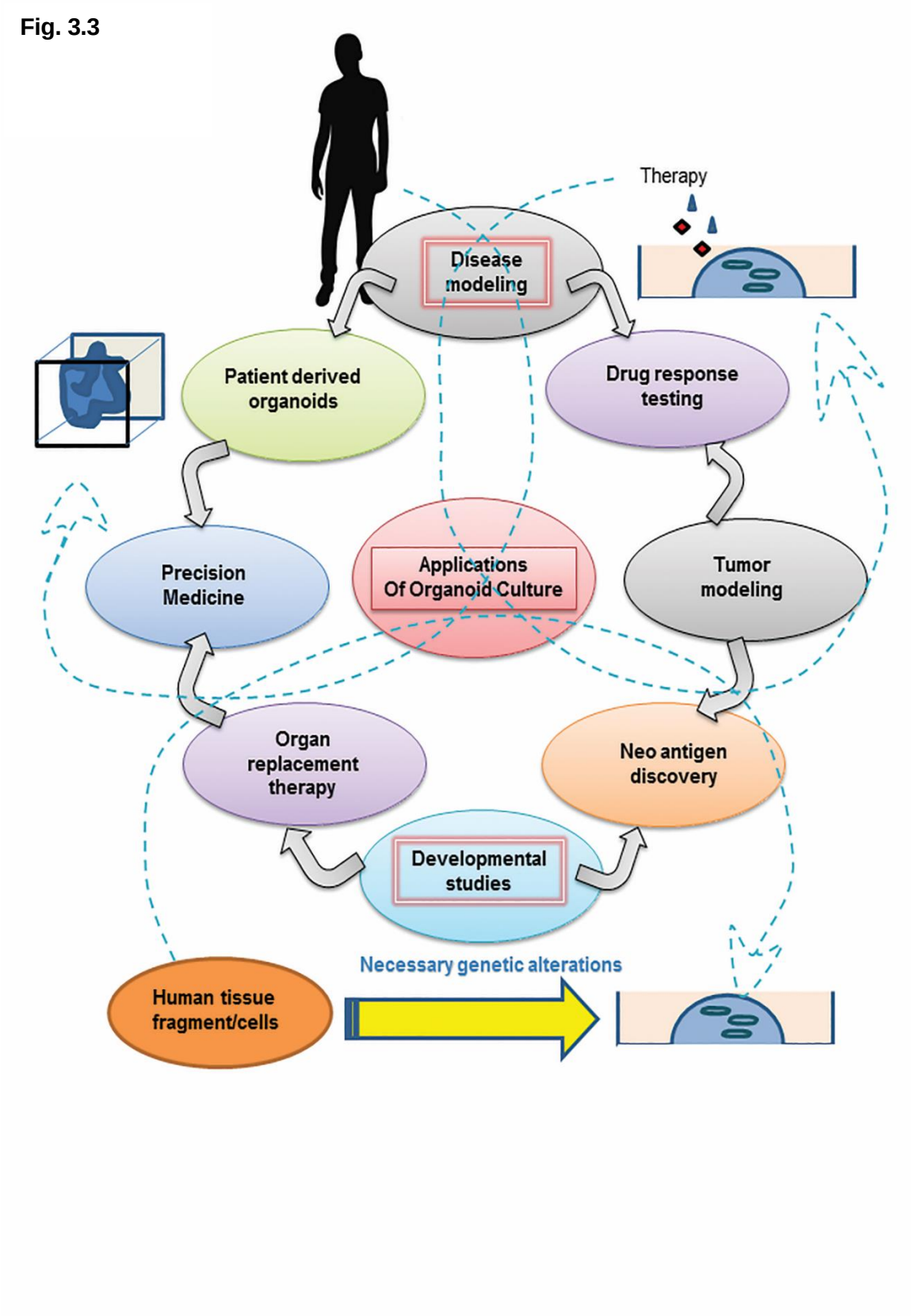

Isolation of organ/tumor
Mechanical digestion
Enzymatic digestion
Cell Collection and Matrigel Embedding
Prostate
Pancreas
Lung
Normal
Tumor
Normal
Tumor
Tumor
Microscopic
Histological
40X
40X
10X
10X
40X
20X
20X
20X
20X
40X

**Fig.3. 3 Potential applications of organoids generated:**

The figure presents prospective applications of organoid culture tool for the advancement of biological research. The arrows in the figure represent the flow of information from tumor modeling, disease modeling and developmental biology studies towards therapeutic interventions. To expand since organoids, represent tissue homeostasis in vitro, they can be used to model pathologies by inducing desired mutations or exposing them to necessary stimulus or pathogens. Following which the pathogenesis and disease development can be studied. Such studies facilitate further research to study drug response or generate organoids directly from patients to device personalized therapeutic strategy as represented by the arrows emerging from disease modeling bubble. Similarly, modeling cancer initiation and progression in organoids can facilitate therapeutic response studies and the discovery of new oncogenic proteins or antigens that can be targeted illustrated by arrows emerging from the Tumor modeling bubble. Additionally, lineage tracing studies or organ development studies using organoids have immense potential for the field of organ replacement therapy and can help neoantigen discovery for cancer research (arrows emerging from developmental biology studies bubble). The dashed arrows represent the overlapping domains amongst these applications as explained above.

**Fig. 3.3**


Therapy
Disease modeling
Patient derived organoids
Drug response testing
Precision Medicine
Applications Of Organoid Culture
Tumor modeling
Organ replacement therapy
Neo antigen discovery
Developmental studies
Necessary genetic alterations
Human tissue fragment/cells

## 3B Generation and optimization of organoid cultures

### 3B.1 Background and rationale

3D culture or organoid cultures provide a robust experimental model that simulates in vivo biology and pathologies and are becoming the popular choice of model to study therapy response (*Kaushik et al. 2018*)**.** These are cell-derived models that simulate endogenous cell organization and architecture hence enable cell behavior, pathobiological and drug response research. Organoids can be generated from very little source material like biopsies, circulating cells isolated from patients granting an opportunity for developing personalized therapy. Further their potential applications in the fields of disease modeling, gene editing, organogenesis, and transplantations makes them instrumental research tools. For these reasons, many studies have attempted to setup cryopreserved biobanks of human organoids as a resource for researchers and clinicians.

In 2009 Sato et al. demonstrated that embedding $LGR5^{+}$ intestinal stem cells into a 3D matrix yield self-organizing organotypic structures revealing a single adult stem cell has the potential to generate organoids [163]. R-spondin1, EGF and noggin were shown to be critical to mimic the in vivo stem cell niche vital for an organoid generation in this study. Further may studies utilized similar niche factor supplementation to create organoids for other multiple mice and human epithelia, including colon, liver, pancreas, prostate, stomach, fallopian tube, taste buds, salivary glands, esophagus, lung, endometrium, and breast [92]. Many studies since have reported stable long-term expansion and cryopreservation of organoids; this makes organoids attractive tools considering lack of available source tissue is a hindrance in

cancer research. Organoids can also be genetically modified and remain genetically and phenotypically stable allowing for a wide range of applications in cancer research.

Huch et al. published the first study reporting conditions for in-vitro expansion of adult bipotent pancreatic progenitor cells through Lgr5/R-spondin axis [164]. A crucial component of their culture medium is the Wnt agonist RSPO1, the recently reported ligand of *Lgr5* and its homologs. Wnt signaling is vital for pancreatic development during embryogenesis but is inactive in adult pancreas [165-167]. An injury to adult pancreas results in reactivation formation of new pancreatic islets in a similar fashion as embryonic pancreas where multipotent progenitor cells give rise to all pancreatic lineages (acinar, duct and endocrine) [168-170]. Partial duct ligation (PDL) or acinar ablation studies have shown that duct cells can proliferate and differentiate towards acinar, duct and endocrine lineages, suggesting presence of pancreatic progenitor pool within ductal cells in adult pancreas [139, 171-175]. Wnt-Lgr5-Rspo signaling axis can be exploited to create culture conditions that enable long-term expansion of adult pancreatic duct cells, with the ability to differentiate towards both duct and endocrine lineages in the presence of proper signals [164].

3D organoids have also been developed from malignant mouse and human pancreatic tissues and utilized to investigate PDAC pathogenesis [93, 137, 176]. Organoids derived from wildtype and genetically modified mouse models PDAC recapitulate disease progression with close physiological relevance in vitro. Additionally, organoids derived from mouse and human PDAC tissues generate PanIN like lesions and progress to invasive PDA, following transplantation. Whereas, organoids from wild-type mouse normal pancreas regenerate normal ductal architecture [137].KC (Kras; PdxCre) and KPC(Kras; p53; PdxCre) mouse models are well established genetically engineered autochthonous mouse models of PDAC and have been used to generate murine PDAC organoids to study therapeutic response [137, 177].

Several studies have reported the development of organoids from stem cells that retain progenitor cells in different organs [102, 163, 164, 178]. Murine pancreatic progenitors grow into 3D complex organoidal structures that spontaneously undergo pancreatic morphogenesis and differentiation when embedded in Matrigel and presence of appropriate medium composition [179]. Recapitulation of in vivo niche signaling interactions is necessary to enable expansion of progenitors, which is achieved by active Notch and FGF signaling [179]. Single isolated adult mouse pancreatic progenitors which belong to ductal lineage have the potential to expand and form organoids in Matrigel-based 3-D culture system [102, 179]. Pancreatic organoids maintain Lgr5 expressing population and can differentiate to endocrine cells [102]. Human and mouse pancreatic organoids allow expansion and the generation of progenitor cell subpopulation. high aldehyde dehydrogenase activity ($ALDH^{hi}$) and expression of pancreatic progenitor markers (*PDX1*, *PTF1A*, *CPA1*, and *MYC*) are maintained in pancreatic organoids [102, 164].

PDAC organoids have been used to study therapy response [159, 180]. Human patient-derived PDAC organoids carry patient specific and histopathologic characteristics and can be used to study the etiology of patient tumor and therapy response. Genetic and structural features of primary pancreatic adenocarcinoma tumors are well represented by human PDAC organoids [181].

We decided to generate 3D culture/organoids from mouse models of pancreatic cancer to study therapeutic strategies and PDAC pathology. We utilized tumors resected from KC and KPC mouse models and wild type mice to generate our organoids. We standardized our protocol and culture conditions by building on previously established culture conditions reported in Sato et. al. We also created organoids from adjacent normal and tumor tissues obtained from PDAC patients to test the effect of our therapy in clinical samples.

Apart from establishing Pancreatic organoids we also developed lung and prostrate organoids in our lab for further utilization in metastatic as well as other studies. In this part of my thesis, we report the generation or organoids and optimization of experimental protocols for normal and tumor organoids. We also report the maintenance of cancer stem cells in our organoids and standardize a method to track and analyze organoid growth and therapeutic response. We use these standardized protocols and methodologies in the next part of this thesis to evaluate our proposed therapy.

## 3B.2. Results

### 3B.2.1 Generation of Organoids

#### 3B.2.1a. Murine Pancreatic Organoids

Pancreas or pancreatic tumors were resected from wildtype C57BL/6, KC, and KPC mice. The resected tissue was mechanically minced to a size of approximately one millimeter. Minced tissue was digested with a mixture of collagenase XI and dispase in DMEM media with the addition of 1% FBS for KPC organoids. A period of 30 minutes to one hour was enough for digestion of wild-type pancreas whereas KC and KPC organoids required 1 hour to 3 hours to digest. During the period of digestion at 30-minute intervals, an aliquot of supernatant from digesting tissue was observed under a microscope. After one and a half hour chains of cells could be observed in the supernatant under the microscope. These chains represent ductal cell population. The supernatant from this digest was taken for organoid culture, and the remaining digest was incubated again for further digestion. The supernatant was taken at several time points until we stopped seeing strings of ductal cells under a microscope. The supernatant was then washed and embedded in growth factor reduced matrigel and seeded in 48 well plates.The seeded matrigel domes were allowed to set for 15 minutes in the incubator.

KC Organoids were supplied with complete AdDMEM/F12 medium supplemented with HEPES, Glutamax, penicillin/streptomycin, B27, N-acetyl-L-cysteine, epidermal growth factor (EGF). Although organoids formed in this media, we got a very low efficiency. Additional growth factor was added for an enhance efficiency namely, fibroblast growth factor 10 (FGF10), Nicotinamide, and A83-01 (TGFb inhibitor), Noggin, R-spondin. However, KPC and normal organoids needed supplementation with FBS for

initial establishment. A recent study reported tumor organoids to appear spherical under a microscope, whereas normal pancreatic organoids appear cystic (*Seino et al. 2018*). Once developed, our tumor organoids grew as single-cell-layered spherical ductal structures *in vitro* and developed more complex structures as the days progressed. Our normal organoids had the cystic appearance and did not gather morphological complexity in a culture like that of HC, KPC organoids (**Fig.3.4a**).

### 3.B.2.1b Human pancreatic organoids

Lack of available human patient tissues is a major hindrance to pancreatic cancer research. Human PDAC organoids have potential in providing ample research material for drug screening and a window for personalized therapy protocol development specific to the patients. Human tumor and adjacent normal samples were obtained from the surgery department and subjected to mechanical and digestion similar to murine organoids with addition of Primocin and A83-01 in digestion media. The digestion and seeding protocol was similar to murine organoids. Human PDAC and normal like organoids needed a further enriched media with the addition of Primocin, gastrin in complete media for both tumor and normal and PGE2 in normal organoids. One representative figure from the 4 successfully generated lines of patient PDAC organoids has been provided in Fig. 2.1b. We could also generate organoids from MUC4 knockout mouse model for PDAC developed in our lab (Fig.2.4C)

### 3.B.2.1c Lung organoids

Lung organoids have typically been derived from isolated progenitor cells from dissociated lung tissue (Hogan et al. 2014). Basal cells exist in the pseudostratified

mucociliary epithelium that lines mist of the conducting airways in the lungs and expands to regenerate the lungs upon injury in adult mouse (Pardo-Saganta et al. 2015). Organoids that represent the mucociliary epithelium of the lungs are generated by seeding isolated basal cells in a matrigel based system (Roch et al. 2011; Tata et al. 2013). Isolated humans pluripotent stem cells (). Bronchioalveolar stem cells (BACs) are club cells that exist in distal bronchioles and have the potential to differentiate into both type 2 Alveolar cells (Stem cells) and airway cells. Organoids that contain both alveolar cell and airway cell lineages can be derived from isolated BACs (). Both the processes have strong requirement of EGF signaling to enable growth. The process of stable human lung organoid development requires stepwise differentiation of pluripotent stem cells from a definitive endoderm state to anterior foregut endoderm and finally to NKX2.1 expressing lung progenitors(). Activation of the hedgehog pathway followed by exposure to FGF 10 signaling allows differentiation to an anterior foregut endoderm state, and further exposure to differentiating factors like BMP signaling allows differentiation into lung progenitors. To generate organoids from a mixed population of cells and to get organoids that represent both proximal and distal epithelium we adapted from the method developed by Biana et al. Lungs from C57BL/6 wildtype and KA ($Kras^{G12D}$; AdCre) and KPA($Kras^{G12D}$; $p53^{R172H+}$ AdCre) were resected and subjected to mechanical and enzymatic digestion similar to pancreatic tissue as reported before. 30 minutes of digestion was enough to allow digestion sufficient for an organoid generation (large clumps of cells were not visible in the supernatant from digesting mixture). The supernatant was then seeded in low attachment plates in presence of BMP, TGFb and Wnt activators, FGF and RA activators allowing expansion of stem cell and progenitor populations. This lead to formation of lung spheroidal structures which were them embedded in Matrigel. Once matrigel solidified the media was replaced to Wnt, FGF, cAMP and glucocorticoids containing media to enable growth of lung organoids. We

successfully generated lung organoids representing two different cancer grades (KA and KPA) (Fig. 2.4D)

**3.B.2.1d Prostate organoids**

Development, homeostasis of prostate and prostate cancer all require active AR signaling (Pienta KJ et al. 2008). Cell line cultures used for prostate cancer study do not have an intact AR signaling pathway. Prostate organoids can be generated by isolated luminal cells, which represent one of the progenitor populations in the prostate (Chua CV et al. 2014). Prostate organoids derived from digested prostate tissues contain multipotent progenitor cells from both luminal and basal lineages and active AR signaling (Drost et al. 2016). Stable AR-responsive and progenitor pool containing prostate organoid expansion require EGF, Noggin, and R-spondin signaling along with AR signaling (Drost et al. 2016). We adapted previously published protocols for prostate, pancreas and intestinal organoids and developed murine wildtype prostate and prostate cancer organoids. Prostate and prostate tumor tissues from wildtype C57BL/6 and Pten conditional knockout mouse was carefully resected avoiding seminal vesicles, remaining urogenital system and fat tissue. The resected prostate was minced into small pieces of around one millimeter followed by enzymatic digestion with Collagenase type II. After 2 hours of digestion supernatant from the digest was mixed with Matrigel and seeded as domes in 48 well plates. Once the matrigel solidified prostate organoid media comprising DMEM/F12, B27, N-acetylcysteine, EGF, Noggin, R-spondin 1, A83-01 and DHT was added to the matrigel domes. We successfully generated organoids from normal prostate and prostate cancer (Fig. 2.4E).

### 3B.2.2 Maintenance of progenitor cells or CSC pool in pancreatic cancer organoids.

Organoids were stained for expression of several self-renewal and CSC markers to examine retention and expansion of CSC population in developed PDAC organoids. Common CSC and stem cell markers utilized in pancreatic cancer were used including OCT4, SOX2, CD44v6, ALDH1, ABCG2, CD133, ESA, and pEGFR to include multiple CSC subpopulation. The beautifully stained organoids confirmed the presence of CSC and self-renewal markers (Fig. 2.5). A recent publication revealed the presence of a new CSC population in PDAC called autofluorescent cells. These cells retain auto fluorescing riboflavin in their ABCG2 expressing vesicles and hence exhibit autofluorescence. We could observe the presence of this newly identified subset of CSC; autofluorescent cells in established organoids (Fig.2.5).

### 3B.2.3 Tracking Growth and therapy response in organoids

We used KC organoids for standardization of the protocols. We could track individual organoids over a span of several days to analyze the change in the size of the organoids over time (Fig. 2.6 a). Our studies revealed that KPC organoids grow significantly faster in culture compared to KC organoids (Fig. 2.6 b). This is an expected growth pattern since KPC mice develop more aggressive tumors as compared to KC mouse. This growth pattern also suggests that organoids retain their in vivo growth pattern in vitro. We could also perform drug treatment and follow organoids to analyze the change in organoids size due to therapy representing growth inhibition (Fig.2.7a,b,c). We use this method of growth analysis in later studies for therapy response analysis.

### 3B.3 Discussion

Developmental pathways employed in embryonic development of pancreas are evolutionarily conserved [93, 137, 163, 182, 183]. Signaling pathways such as Wnt, BMP, FGF, HGF, EGF, RA and TGFb are some of the vital pathways responsible for generating a morphological gradient that allows development of the pancreas. The standard requirement to enable the growth of pancreatic 3D cultures is to supply factors to enable the functionality of these pathways since they promote proliferation, survival, and migration of progenitor pools [92]. To provide a supportive ECM which also allow cell motility we used matrigel as reported by several other studies. To supply necessary nourishment, we try multiple media compositions with limiting growth factor availability and find a difference in efficiency of organoids generation. This suggests a requirement of an enriched media for high-efficiency organoids generation but the possibility of utilizing a growth factor limiting media to facilitate the growth of organoids as per the need of the experiment. A recent study has reported limiting the availability of EGF and Noggin to allow enrichment of KRAS mutant and SMAD 4 mutant organoids [184]. Previous studies from other labs have reported the development of pancreatic tumor organoids that retain attributes of pancreatic ductal adenocarcinoma from duct cells isolated from neoplastic tissues [137]. We mince and enzymatically digest pancreatic tissues enough to isolate chains of ductal cells.

Different enzymes and duration of digestion were required for tissues from different origins such as the normal murine or human pancreas, and KC, KPC, and patient pancreatic tumors. It is critical to achieving appropriate digestion of the tissue since too much or too little digestion would both lead to failure of the protocol. Over-digestion damages the ductal cells whereas under digestion leaves them in clumps that

have cells from acinar and ductal cells and presence of acinar cells limits ductal cell growth.

### 3B.4 Figures and Legends

**Fig. 3.4 Established KC, KPC ad Human PDAC organoids**

Organoids were generated from respective murine or human tissues following enzymatic digestion and matrigel embedding. After a week in culture murine organoids were observed under microscope and images were clicked. Human organoids were cultures for 3 weeks before the pictures were clicked. Organoids were subsequently paraffin embedded and sectioned. The slides were then stained with hematoxylin and eosin to observe structural morphology of the organoids. A) Microscopic and Hand E stained representative images of established mouse KC, KPC and wildtype organoids. B) Microscopic and Hand E stained representative images of established human PDAC organoids. C)Microscopic and Hand E stained representative images of established MUC16 Ko KPC organoids. D) Microscopic and Hand E stained representative images of Prostate Normal and Cancer organoids. E) Microscopic and Hand E stained representative images of established Lung Cancer Organoids

**Fig. 3.4**

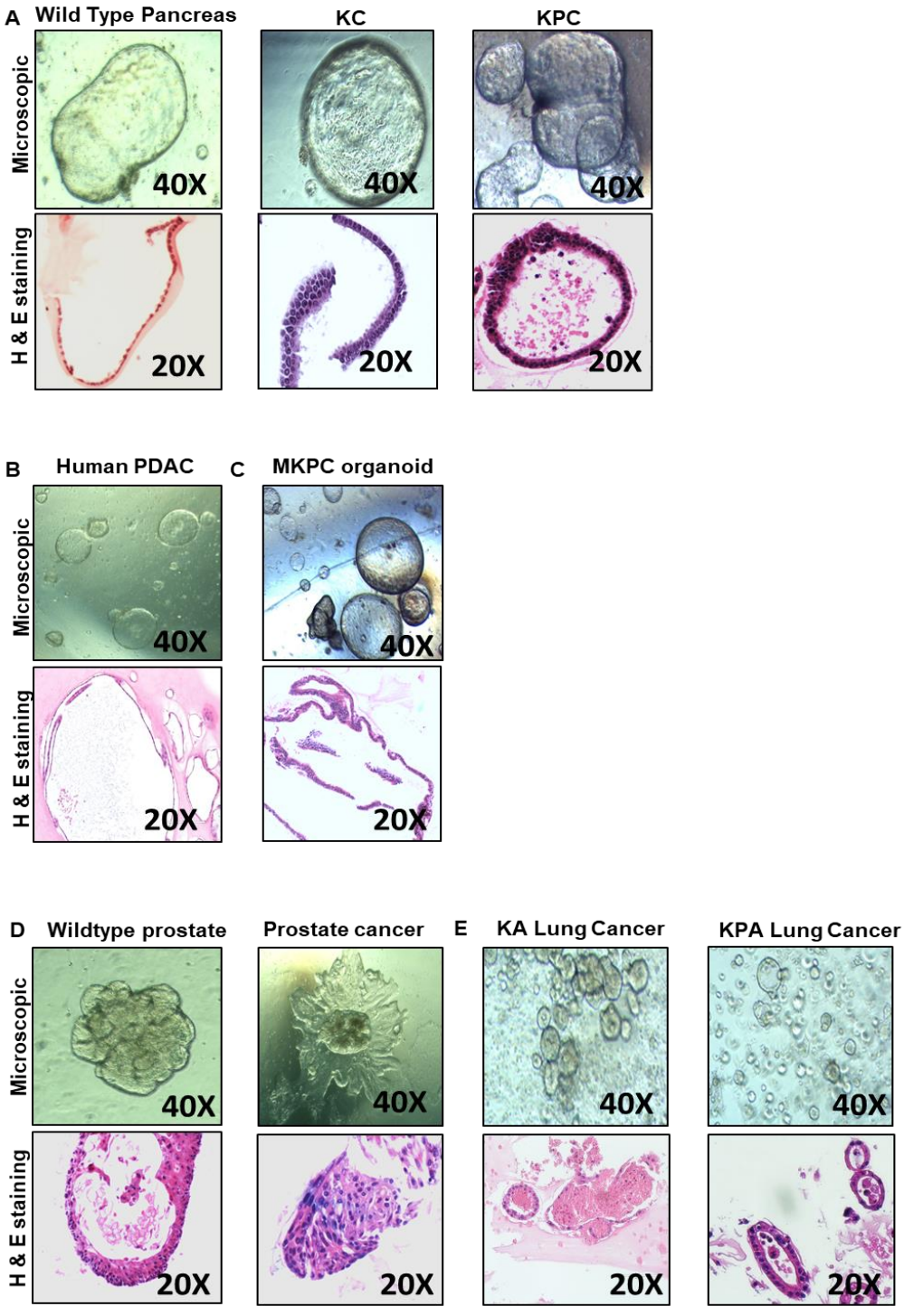

A
Wild Type Pancreas
KC
KPC
Microscopic
H & E staining
40X
20X
B
Human PDAC
C
MKPC organoid
D
Wildtype prostate
Prostate cancer
E
KA Lung Cancer
KPA Lung Cancer

**Fig. 3.5 CSC population is maintained in pancreatic cancer organoids**

Organoids were paraffin embedded and sectioned. The slides were then stained with respective fluorescently stained antibodies and DAPI to observe molecular expression. A) Immunofluorescent stained images of pancreatic organoids stained for CSC and self-renewal markers CD44, ALDH1, ABCG2, CD133, OCT4, SOX2, ESA and pEGFR. This suggests presence of multiple cancer stem cell subpopulations in our organoids including but not limited to CD133 expressing, ALDH1 expressing and CD44 expressing populations that have widely studied in PDAC. B) DAPI stained organoids with autofluorescent cells. Organoids were paraffin embedded and sectioned. The slides were then DAPI stained to observed presence of any autofluorescent cells. Autofluorescent cells represent retention and expansion of another recently identifies cancer stem cell subpopulation in pancreatic cancer.

**Fig. 3.5**

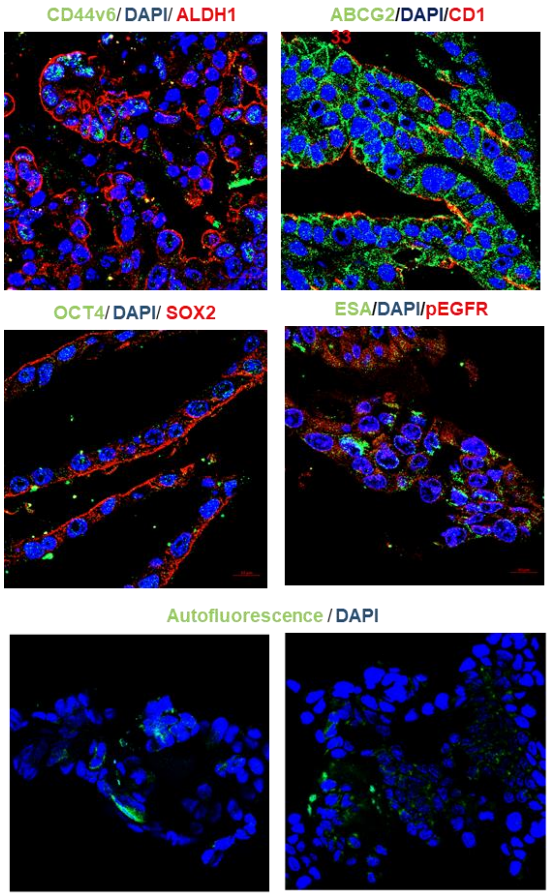
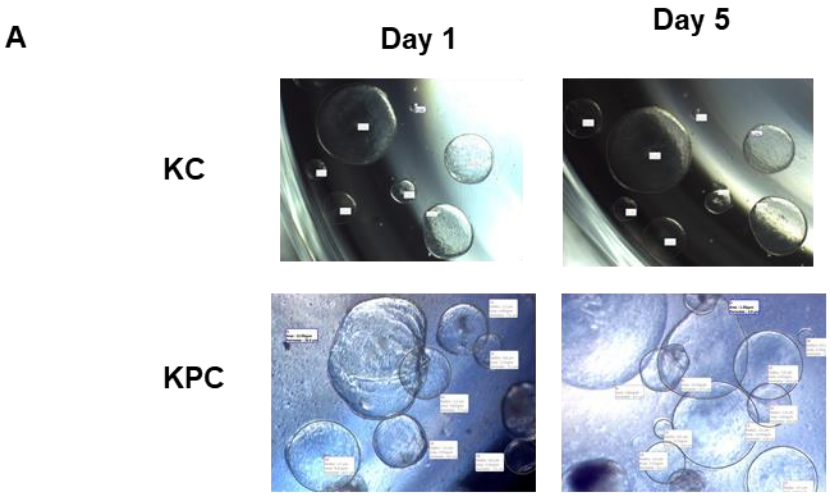

CD44v6/ DAPI/ ALDH1
ABCG2/DAPI/CD1
33
OCT4/ DAPI/ SOX2
ESA/DAPI/pEGFR
Autofluorescence / DAPI

**Fig. 3.6 Organoids growth in culture**

KC and KPC organoids were embedded in matrigel domes and seeded in 48 well plates After a two days in culture ten KC and KPC organoids were observed under microscope and images were clicked from multiple fields. The wells containing these organoids were then marked for the location of these organoids. For the next five days the location and size of these organoids were tracked/followed. At the end of five days pictures were clicked again for the tracked organoids. A) Microscopic image of KC and KPC pancreatic organoids that have been tracked over 5 days representing change in their size B) Quantification of change in the size of organoids from day 0 to day 5 to examine their growth in culture. The sizes of tracked organoids were measured by clicking their picture and measuring each organoid using the Moticon Software. Size change of the organoids from day 0 to day 5 was then calculated. KPC organoids grew at a higher rate in culture compared to KC organoids revealing their aggressiveness ($P<0.005$).

**Fig. 3.6**


A
Day 1
Day 5
KC
KPC

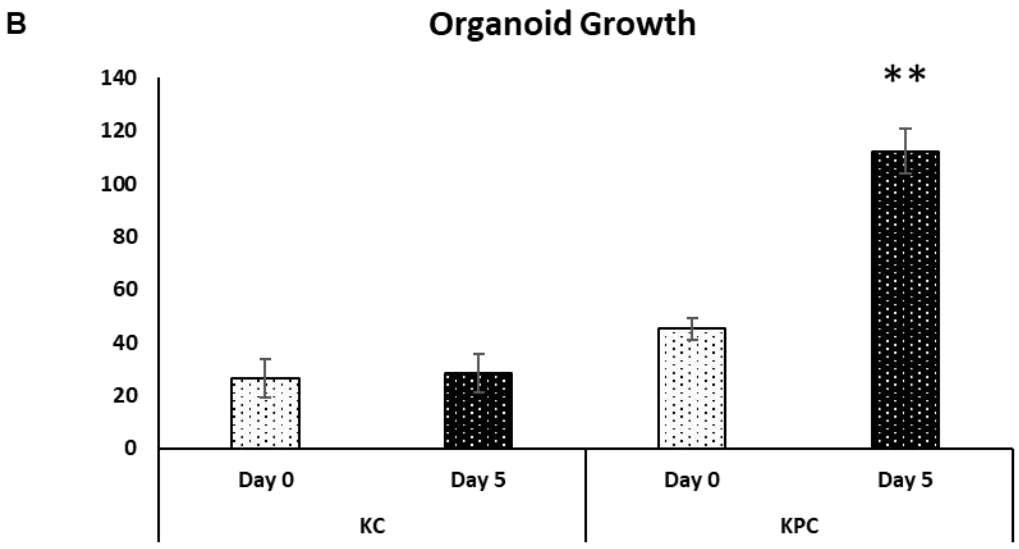
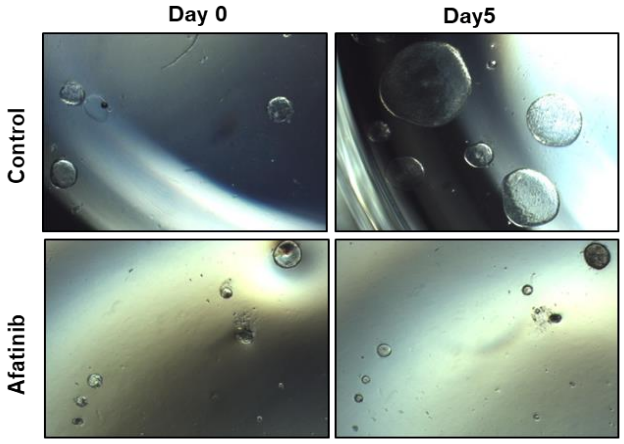

B
Organoid Growth
140
120
100
80
60
40
20
0
**
Day 0
Day 5
Day 0
Day 5
KC
KPC

**Fig. 3.7 Growth inhibition tracking in organoids**

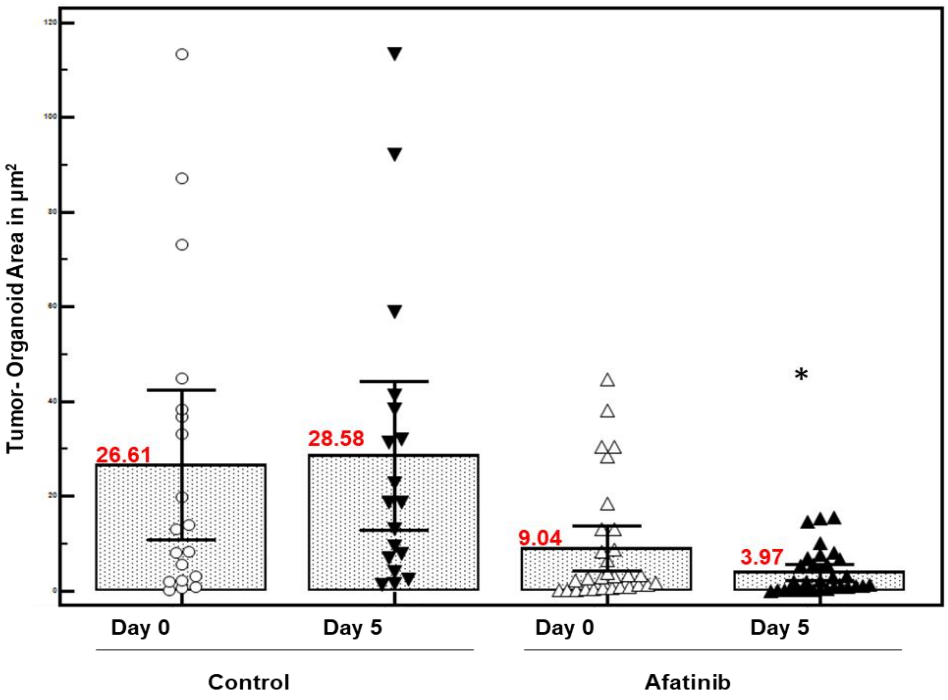
KPC organoids were embedded in matrigel domes and seeded in 48 well plates in two groups “control” and “Afatinib” (A small molecule pan-EGFR inhibitor approved from NSCLC). After two days in culture both groups of organoids were observed under microscope and images were clicked. The wells containing these organoids were then marked for the location of these organoids. Afatinib was added at a concentration of 0.1μM in group named “Afatinib” and control group was given equal microliters of PBS. For the next five days the location and size of these organoids were tracked/followed. Media was changed on day 2 and 4 for both groups with re-administration of Afatinib and PBS. At the end of five days pictures were clicked again for the tracked organoids. A) Microscopic image of pancreatic organoids for day 0 and day 5 in the two groups (Untreated “control”, Drug-Treated” Afatinib”). B) Quantification of change in the size of ten organoids in control and drug-treated group from day 0 to day 5. The sizes of tracked organoids were measured by clicking their picture and measuring each organoid using the Moticon Software. The average size in the group is written in red over each bar. Evaluation of % size change in organoids upon drug treatment to evaluate therapy response. Therapy response was evaluated by comparing change in organoids over 5 days in the two groups. Control organoids showed in average 7% increase in their size (area). Drug-treated organoids decreased in size by 56% suggesting successful inhibition or growth due to therapy($P<0.5$).

**Fig. 3.7**

A




B




C

| | Day 1 | Day 5 | % increase | % decrease |
|---|---|---|---|---|
| Control | 26.61579 | 28.58889 | 7.413267 | -7.41327 |
| Afatinib | 9.04 | 3.974194 | -56.0377 | 56.03768 |

# CHAPTER 4: PANCREATIC CANCER STEM CELL TARGETING: AFATINIB INHIBITS EGFR/FOXA2/SOX9 AND DECREASE STEMNESS PROPERTY

## 4.1 Introduction

Improving disease-free survival and a patient's quality of life is the comprehensive goal of research against PC. Although several therapies have been tried, none has significantly improved patient outcomes. The present therapeutic options for advanced metastatic PC primarily include gemcitabine and its combination with erlotinib or nab-paclitaxel or FOLFIRINOX [7]. Despite all the available therapies and surgical interventions, the average five-year survival rate for PDAC patients is merely 8% [7]. The primary cause of PC mortality is metastasis. Approximately 53% of patients upon diagnosis present with an advanced metastatic stage of the disease [7]. Furthermore, emerging evidence suggests that PC is metastatic even at its conception, which makes targeting this aggressive metastatic cancer a challenging task [5]. One of the major factors contributing to metastasis is mobilization of CSCs [185, 186] a small subpopulation of cells in the tumor also referred to as side population (SP) which has been shown to be vital for PC metastasis and drug-resistance [2, 187]. These cells are also responsible for drug resistance and disease relapse in PC and other types of cancers [2]. Conventional chemotherapeutic drugs act on differentiated tumor cells but fail to target the CSC population. It is now clear that the success of new PC therapies in achieving durable remissions will depend on targeting CSCs; therefore, understanding mechanisms for targeting PCSCs is critical.

The EGFR family of proteins are involved in the initiation and progression of PC, with EGFR mutations being essential for the initiation process [188]. Many ongoing clinical trials are using inhibitors against EGFR family of proteins for therapy against cancers. The USFDA recently approved one such inhibitor erlotinib in combination with gemcitabine for the treatment of advanced PC; however, later this treatment caused dose-limiting toxicity and failed to control the compensatory changes in the

phosphorylation of HER3, resulting in resistance against erlotinib [189, 190]. Afatinib is a third-generation EGFR family inhibitor and has been shown to be superior in inhibiting PC cell lines when compared to erlotinib [190]. It is an FDA-approved drug for non-small cell lung carcinoma and is being investigated for several other cancers [191, 192].It is a pan-EGFR inhibitor (targeting all EGFR family proteins) and is presently being investigated as a single agent and in combination with other chemotherapeutic drugs in 49 clinical trials; however, few studies have examined its effects on CSC populations in different cancers [193]. Our previous study has shown that afatinib can be used to inhibit EGFR proteins in vitro and reduce colony formation and invasion of PDAC cell lines [194].

Here we tested the potential of afatinib in specific targeting of the PCSCs along with differentiated cancer cells. We identified that afatinib inhibits a novel EGFR/ERK/FOXA2/SOX9 axis and abrogates PCSC. Through inhibition of PCSC the combination therapy of afatinib with gemcitabine inhibits PC as well as its metastasis.

## 4.2 Results

### 4.2.1 High self-renewal marker expression in Gemcitabine treated and high-grade pancreatic cancer.

The present standard of care for pancreatic cancer patients is either neoadjuvant chemotherapy or chemotherapy depending on the spread and grade of the disease. Cancer stem cells have been shown to remain intact even upon standard chemotherapeutic treatments in pancreatic cancer [195]. Additionally, some recent studies have suggested CSC enrichment due to chemotherapeutic drugs [196, 197]. With an objective to examine the presence of CSC population in PDAC patients, we immunofluorescent stained PDAC tissue microarray for the presence of CSC and self-renewal markers like SOX9, CD44, ESA and CD133 (Fig. 4.1A). We observed enhanced expression of these markers in PDAC tissues compared to normal pancreatic tissue. Upon segregating tissue samples according to patient grade, we found an increase in these markers as the grade and severity of PDAC increases (Fig. 4.1A,B). There was a significant increase in expression of these markers from normal pancreatic tissue to PDAC stage II grade 2 malignant tissue and finally to PDAC stage III grade 3 malignant tumors. Amongst these markers, SOX9 is known to be a master regulator for several stem cell genes in the pancreas as well as a valuable marker for the ductal lineage of pancreatic cancer cells [198, 199]. To examine how chemotherapeutic treatments, affect PCSCs we immunohistochemically stained human PDAC tissue microarray for SOX9 expression. The stained slides were scored with the help of a pathologist. A subset of PDAC tumors with a history of chemotherapeutic treatment were found to express a significantly higher amount of SOX9 compared to patient samples with no history of chemotherapy (Fig. 4.1 C). Although when the collective of all samples were compared the difference in SOX9 expression was not significant which can be attributed to

extensive intertumoral heterogeneity that exists in PDAC. High expression of CSC markers in PDAC samples and patient samples with a history of chemotherapeutic treatments suggests the role of PCSCs in disease aggressiveness and a probable enrichment upon chemotherapy.

### 4.2.2. Development and characterization of murine PC organoids

Pancreatic tumor organoids developed from neoplastic pancreatic duct cells were previously shown to retain the same attributes as the pancreatic ductal adenocarcinoma (PDA) stage [137]. Therefore, to examine the treatment efficacy of afatinib and gemcitabine on PC, we generated organoids from pancreatic tumor tissues of KC and KPC mouse. (Fig. 4.2A). Once developed, they grew as single-cell-layered ductal structures in vitro and developed more complex structures as the days progressed (Fig. 4.2B, Fig. 4.2C). 3-D tumor organoids were used since they are more physiologically relevant than traditional monolayer cultures to test therapy response [93].

Afatinib and gemcitabine inhibit the growth of tumor organoids derived from KC, KPC mice, and human PC tumors.

EGFR family of proteins are implicated in multiple cancers including PC [200]. In PC, the presence of EGFR in serum has been shown to be correlated with poor prognosis [201], and inhibition of EGFR via erlotinib has shown survival benefits for PC patients [202]. Once developed, KC and KPC organoids and human PC tumor (Supp. Fig. 1A and Fig. 1A, B and C) were treated with afatinib alone and in combination with gemcitabine. Results showed that KC and KPC control organoids left untreated grew in size by 45% and 60% respectively (Supp. Fig. 1B, C and Fig.4.2D, E,) which we attributed to their normal growth. Upon afatinib treatment, KC and KPC organoids decreased in size by 25% and 16% (Supp. Fig. 1B. C and Fig. 4.2D, E); we also observed an increase in the

size of KC and KPC organoids by over 50% on treatment with gemcitabine (Supp. Fig. 1C, D). Since the growth of these organoids has been linked to their ability to maintain progenitor cells or stem cells [203], we presume that the increase in the size of the organoids is due to enrichment of CSCs upon gemcitabine treatment. Our results showed combination treatment with afatinib and gemcitabine decreased KC organoid size by 57% (Supp. Fig. 1B, C), suggesting a potential synergistic effect of the combination in inhibiting PDAC.

Interestingly, the concentration of afatinib (0.1 μm) employed in KC organoids was not sufficient to restrict the growth of KPC organoids (Supp. Fig. 1F). We found that an increased concentration of afatinib (0.5 μm) with gemcitabine (1 μm) inhibited KPC organoid growth and size by over 50% in comparison to untreated control, which grew in size and complexity. In both KC and KPC organoids, the untreated KPC control organoids evolved from single-cell-layered ductal structures to more complex cribriform structures, whereas afatinib restricted this growth (Fig. 4.2E, and Supp. Fig. 1C). The combination also visibly eradicated complex organoid structures as revealed by H&E-stained sections (Fig. 4.2E, and Supp. Fig. 1C), which suggests the therapy could reduce the aggressiveness of PC. Over 40% of organoids remaining after seven days of consecutive combination treatment in both KC and KPC organoids were broken, showed no structural complexity or were eradicated or dead (Supp. Fig. 1D, E).

Further, we developed human pancreatic tumor organoids (Fig. 4.2C) to test the efficacy of afatinib and gemcitabine and their combination. Treatment with afatinib significantly reduced the size of human pancreatic tumor organoids compared to control, whereas the gemcitabine-treated organoids did not show any significant variation in size (Fig. 4.2F, G). However, the combination of afatinib and gemcitabine treatment significantly reduced pancreatic tumor organoids size (Fig. 4.2F, G). Overall, these results suggest

that afatinib alone and in combination with gemcitabine can decrease tumorigenesis and growth in PC.

### 4.2.3. Combination of afatinib and gemcitabine significantly inhibits PC cell growth and in vivo primary tumor and metastasis

To further determine the effects of afatinib and gemcitabine combination treatment on PC cell lines, SW1990 and Capan1 cells were treated with different concentrations of afatinib and gemcitabine for 24 h, and the IC50 value was calculated using MTT analysis (Supp. Fig. 2A). To evaluate the inhibitory effect of afatinib and gemcitabine on the EGFR family of proteins, SW1990 and Capan1 cells were treated with afatinib and gemcitabine alone and in combination for 48 h. We found that treatment with afatinib alone and in combination with gemcitabine effectively downregulated the phosphorylation of EGFR, Her2, and Her3, with no change to their total protein expression (Fig. 4.3A). However, treatment with gemcitabine alone leads to increased phosphorylation of EGFR family proteins when compared to afatinib treatments. Furthermore, we tested the combination therapy in vivo in xenograft nude mouse model of PC. For this, athymic nude mice were orthotopically injected with luciferase-labeled Capan1 cells into the head of the pancreas, and then divided into four groups (7 mice per group): control (PBS), afatinib (orally), gemcitabine (intraperitoneal) and the combination of both (Fig. 4.3B.) Afatinib treatment reduced the primary tumor burden by over 1.8-fold (Fig. 4.3C, D) and reduced the number of metastasis spots per organ (Supp. Fig. 2B); afatinib also decreased the metastatic incidence to the liver by 16-fold, mesenteric lymph nodes and intestine by 8-fold and diaphragm and stomach by 4-fold when compared to control (Fig. 4.3E, F). A combination of afatinib and gemcitabine reduced the primary tumor by 2.4-fold compared to 1.5-fold by gemcitabine alone (Fig. 4.3C, D). The combination also decreased the incidences of metastases by 16-, 4-, 32-,

8- and 4-fold in the liver, mesenteric lymph node, diaphragm, and stomach, respectively (Fig. 4.3E, F). Taken together, these results suggest that afatinib treatment in combination with gemcitabine can improve the anti-tumor effect of gemcitabine in PC.

### 4.2.4. Afatinib decreases the SP/CSC population and tumorsphere formation in PC cells.

The decrease in tumor weight and metastasis in the xenograft mouse model encouraged us to analyze the PCSC population because of its association with metastasis, poor prognosis, and drug resistance in PC [204] . Hoechst-based FACS analysis was used to analyze and isolate the CSC population as described in Materials and Methods (Fig. 4.4A). We next measured the drug sensitivity (IC50 values) of PCSC cells for determining an effective drug dosage (Supp. Fig. 2A). SP cells were found to be more sensitive to afatinib: 46.93% in Capan1 and 56.7% in SW1990 in comparison to their parental populations. We also report that the SP fraction is more resistant to gemcitabine (27.45% in Capan1 and 26.4% in SW1990 of parental populations) when compared to their parental counterparts. Based on these results, we suggest that the SP fraction is more sensitive to afatinib and resistant toward gemcitabine (Supp. Fig. 2A). To further evaluate the effect of afatinib on CSC/SP populations, PC cells were subjected to afatinib, gemcitabine and the combination treatment of both the drugs for 48 h and then stained with Hoechst 33342 (a fluorescent DNA-binding dye) to analyze the CSC/SP cells by FACS analysis [88, 205]. Afatinib and combination treatment significantly decreased CSC/SP cells when compared to control cells; however, gemcitabine treatment increased the proportion of CSC/SP cells (Fig. 4.4B, C, D, E). This result suggests that afatinib is effectively inhibiting the CSC population while gemcitabine is enriching it. To further validate the effect of afatinib on PCSCs, we performed immunofluorescence analysis on SW1990 and Capan1 cells and found that treatment

with afatinib inhibits the expression of CSC markers such as ESA, ALDH1, OCT4, and SOX9 along with pEGFR (Supp. Fig. 3A and B) in PC cells. We also examined the effect of afatinib on self-renewal and tumorigenic potential in CSCs using tumorsphere assay on the isolated SP. Treatment with afatinib for 48 h significantly reduced the number of tumor spheres (Fig. 4.4F, G), which further indicates that afatinib affects tumorigenic and self-renewal properties of SP cells.

### 4.2.5. Treatment of afatinib significantly decreases the CSC markers in tumor organoids and in pancreatic xenograft tumors

Our data demonstrated that afatinib acts on PCSCs and reduces their stemness and has a pronounced anti-cancer effect when given in combination with gemcitabine. Therefore, we next asked whether afatinib can downregulate the CSC markers in KPC organoids or in the xenograft tumors. KPC tumor organoids were sectioned and immunostained for CSC markers such as CD44v6, ALDH1, CD133, ABCG2, OCT4, SOX2, and ESA, along with pEGFR. Confocal imaging of KPC organoids shows afatinib treatment drastically reduced CD44v6, ALDH1, CD133, ABCG2, OCT4, SOX2, ESA and pEGFR, which were further decreased by treatment with afatinib and gemcitabine combined compared to the untreated control and gemcitabine-treated groups (Fig. 4.5 A, B and Supp. Fig. 4A). To determine whether afatinib might also affect the CSC markers in vivo, we fluorescently stained the pancreatic tumors resected from the xenograft experiment for CSC markers, i.e., CD133, CD44v6 ESA and pEGFR (Fig. 4.5C, D). Afatinib treatment alone and in combination with gemcitabine decreased CSC markers in primary tumors, suggesting that afatinib is inhibiting PCSC stemness by downregulating CSC markers. Expression of CD44v6 and CD133 has been shown to be critical drivers of PC metastasis [206-209], and ESA is a well-known marker for PCSCs [210] Reduction in these markers further demonstrates the efficacy of afatinib against PCSC. Interestingly, we also observed an

increase in the same markers with gemcitabine treatment, which corroborated our previous data suggesting gemcitabine enriches CSCs (Fig. 4.5 C, D). Since we saw a decrease in primary tumor weight, we wanted to analyze the correlation of primary tumor weight with metastatic incidence. Primary tumor weight correlated poorly with metastatic incidence (Supp. Fig. 2C). These results suggest that a decrease in CSC markers by afatinib may have led to a decrease in metastatic incidence.

### 4.2.6. Afatinib abrogates CSC markers and oncogenic signaling in CSCs

We next attempted to further explore the action of afatinib on self-renewal and CSC markers in PCSCs. As expected, afatinib alone and in combination with gemcitabine decreased self-renewal and CSC markers ALDH1, CD44, SOX2, SOX9, ESA, and ABCG2 and inhibited activation of oncogenic proteins such as ERK and FAK (Fig. 4.6A). To further validate our observation, we first isolated the SP and the NSP cells from SW1990 as described above, and characterized their CSC markers; SP-SW1990 expressed higher amounts of SOX2, PAF1/PD2, ESA, ALDH1, SOX9, CD44v6, ESA and SHH than its NSP counterparts (Fig. 4.6B). The SP-SW1990 cells were then subjected to IC25 and IC50 concentrations of afatinib, which decreased expression of CD44v6, ESA, SOX9 and ALDH1 along with pEGFR and pHer2 (Fig. 4.6C). Next, we performed immunofluorescence analysis on the SP cells (isolated from SW1990 and Capan1) and observed downregulation of ALDH1, ESA, SOX9 and OCT4 upon afatinib treatment (Fig. 4.6 D, E, F, G). These results suggest that afatinib inhibits PCSC by down-regulating molecules vital for CSC maintenance and self-renewal and hence inhibiting stemness in CSCs.

### 4.2.7. Afatinib decreases CSC populations via ERK/FOXA2/SOX9 signaling in PCSCs.

To delineate the mechanism of action of afatinib, we performed a PCR array comparing vital transcription factors and CSC markers in SW1990 cells (Fig. 4.7A). SW1990 cells were treated with afatinib (IC50) for 4 days, followed by RNA isolation and PCR array analysis. Afatinib treatment led to a greater than 2-fold decrease in 23 transcription factors (ABCB5, ALCAM, BMP7, CD38, CD44, DACH1, DLL4, FOXA2, ITGA2, JAG1, KLF4, MERTK, NOS2, PECAM1, PLAT, PLAUR, PTPRC, SNAI1, TAZ, TWIST2, ACTB, B2M and HGDC) important for CSCs (Fig. 4.7A). These results were further validated by qPCR and these genes were found to be downregulated upon four days of treatment (Supp. Fig. 5C). We focused on FOXA2 because of its importance in promoting self-renewal of pancreatic progenitor cells [211, 212]. FOXA2 is involved in regulation of SOX9 expression, which is another self-renewal marker for PCSC[212]. We performed qRTPCR analysis for FOXA2 and SOX9, which showed a decrease in mRNA levels upon afatinib treatment on SP-SW1990 (Fig. 4.7B) suggesting a probable mechanism of action. A scanning of the SOX9 promoter region for binding motifs for FOXA2 revealed eight possible binding sites (Supp. Fig.5A) are the further corroborating possibility of FOXA2 regulating SOX9. It has been reported previously that the phosphorylated form of ERK regulates FOXA2 transcription, and hence we examined pERK levels. As expected, afatinib treatment decreased pERK levels in PC cell line as well as in the isolated SP/CSC population, suggesting that afatinib inhibits PCSCs by inhibiting EGFR/ERK /FOXA2/SOX9 signaling (Fig. 4.7D, E).

To confirm this mechanism, we transiently knocked down EGFR (Fig. 4.7C) in isolated SP/CSCs. Results showed that EGFR knockdown led to inhibition of pERK, SOX9 and FOXA2 expression along with multiple other CSC markers such as OCT4, NANOG, and

DCAMLK1 (Fig. 4.7C), confirming that the decrease in CSC markers and the downstream signaling was due to EGFR inhibition. To further confirm the proposed mechanism, we used two independent siRNAs to downregulate FOXA2 mRNA, which led to a decrease in SOX9 expression, further corroborating the signaling axis (pEGFR/pERK/FOXA2/SOX9) that is modulated by afatinib. To determine if afatinib engages the same mechanism in organoids and xenograft tissues, KPC organoids and xenograft sections were stained with pERK, FOXA2, and SOX9. The results showed that afatinib decreased expression of these markers (Fig. 4.7G, Supp. Fig 4B, C, and Supp. Fig. 5B, C, D). Altogether, our data provide a novel mechanism of action by which afatinib inhibits the PCSCs in PC (Fig. 4.7H).

### 4.3 Discussion

PC is a devastating disease with an abysmal prognosis [7, 213]. Current treatment options that provide a survival advantage for advanced metastatic PDAC include irinotecan liposome injection, gemcitabine, erlotinib, FOLFIRINOX, nab-paclitaxel, and combinations of these drugs. Such available therapies offer a 5-year survival rate of merely 8% and an average overall survival benefit of 5.6 months to 11 months [214]. Aggressive and early metastasis is the chief cause of mortalities in PDAC, and present evidence supports that PDAC may start disseminating as early as its inception, accounting for the dismal prognosis [5, 193, 210, 215]. The cells that likely initiate early metastasis are CSCs, which have the potential to seed tumors, promote metastasis and provide drug resistance [193, 210, 215].Targeting CSCs may provide an opportunity to impede tumor growth and metastasis in PDAC; however, currently used therapies fail to inhibit these cells [7, 193, 216]. We report an increase in expression of CSC markers in PDAC as the grade and severity of PDAC increases which is potentially unaffected by chemotherapy. These results highlight the importance of developing targeted therapy directed towards the CSC population in PDAC. Our study outlines a strategy to effectively impede CSCs in PDAC by inhibiting their maintenance and stemness properties.

As a strategy to target pancreatic CSCs, we tested combination therapy of pan-EGFR inhibitor, afatinib with first-line therapeutic drug gemcitabine. PDAC is known to overexpress EGFR family members HER1 (40-70%) and HER2 (22%) [194], which are vital for the self-renewal and maintenance of CSCs in PC [216]. Inhibition of EGFR family proteins has been reported to downregulate tumor growth and metastasis in multiple studies of PDAC. Furthermore, afatinib inhibits all four EGFR family members by binding to their ATP binding domain and inactivating them [217]. Although afatinib is

an FDA-approved drug for non-small cell lung carcinoma, emerging evidence suggests its role as a potent inhibitor of multiple tumor types [190, 191, 217]. In this study, we illustrated that inhibition of EGFR family of proteins using afatinib significantly reduced the stemness properties of pancreatic CSCs and provided antitumor activity against PC that was enhanced with gemcitabine treatment. Similarly, several other studies have reported that EGFR family inhibition suppresses CSCs in brain, lung and breast cancers, amongst others [218-220]. In addition, our study has shown that pan-EGFR inhibition downregulates pancreatic tumor growth in xenograft mouse models.

To test the effect of afatinib in PDAC models, we developed 3-D tumor organoids from KC and KPC autochthonous mouse tumors, which show clear progression structures similar to PDAC progression. Emerging studies have substantiated the use of tumor organoids for therapeutic response studies because of their high physiological relevance [93] to the tumor. Tumor organoids recapitulate tumor architecture and biology in vitro [137] and maintain a robust population of CSCs, making them ideal for our study. Treatment with afatinib alone or with gemcitabine drastically inhibited the tumor size and architecture in both models of tumor organoids as compared to gemcitabine alone, demonstrating its effectiveness in impairing tumor growth. This was in concordance with the reduction in tumor burden and metastatic incidence compared to gemcitabine alone as observed in our xenograft mouse model experiments. These results also align with our previous publication depicting a reduction in tumor burden upon inhibition of EGFR family proteins by canertinib [194]. The observed increase in tumor organoid size and structural complexity upon gemcitabine treatment, although not significant, may stem from the enrichment of pancreatic CSCs, since CSCs are resistant to gemcitabine [215].

Interestingly, afatinib alone and in combination with gemcitabine decreased distant metastatic spread suggesting afatinib could be effective in inhibiting the metastasis in PDAC patients. This potential is supported by various other reports of

metastasis being linked to the overexpression and amplification of EGFR and its other family members HER2, HER3 and HER4 in several primary cancers like colorectal, ovarian, non-small cell lung carcinoma and pancreatic cancer [221-224].CSCs are known to be vital for metastasis in multiple cancers including PC. Thus, we decided to analyze the effect of afatinib on the CSC population. Our data revealed that afatinib significantly reduces the SP/CSC population in PDAC cell lines and inhibits their self-renewal and tumorigenic potential. Furthermore, our immunofluorescence staining results showed that afatinib treatment downregulated the expression of pEGFR and CSC markers such as CD44v6, ALDH1, ESA, and CD133 in PC organoids and xenograft tumor tissues.

In our study, we delineated a novel mechanism by which afatinib selectively inhibits CSCs in PC. Afatinib treatment reduced several CSC markers along with oncogenic signaling molecules like ERK and FAK. Additionally, we found afatinib downregulated 22 vital transcription factors and CSC markers by more than two-fold compared to control. Among them were several genes essential for PCSC maintenance like KLF4, CD44, SNAIL1, TWIST2, FOXA2, and TAZ. FOXA2 caught our attention because of its role in stem cell self-renewal [225]. It is also vital for lineage specification in the pancreatic formation, and its expression is essential for pancreatic progenitor cells. FOXA2 is also known to regulate SOX9 [212] Both SOX9 and FOXA2 were downregulated by afatinib treatment, and availability of FOXA2 binding motifs in SOX9 proximal region suggested a probable mode of action. SOX9,is known to be vital for CSC self-renewal and is a master regulator for several stem cell markers, is also regulated by pERK [226] which is a downstream molecule of EGFR. This led us to hypothesize that afatinib is targeting the EGFR/ERK/FOXA2/SOX9 axis to inhibit PCSCs and their stemness properties. Our hypothesis was confirmed when knockdown of EGFR decreased expression of FOXA2 and SOX9 and knockdown of FOXA2 decreased

expression of SOX9. To further verify the mechanism of action, we stained afatinib-treated tissue and organoid sections with fluorescent antibodies against pERK, SOX9, and FOXA2, and observed decreased expression of these stemness molecules. Overall, these results affirm that afatinib inhibits CSC maintenance and proliferation via EGFR/ERK/FOXA2/SOX9 inhibition in PC, thereby decreasing PC growth and metastasis

In conclusion, afatinib treatment alone decreased the CSC/SP fraction in PC models by altering their self-renewal/tumorigenic potential. Afatinib, unlike gemcitabine, reduced tumor organoid size and architecture and inhibited CSC markers within these organoids. Furthermore, combination therapy with afatinib and gemcitabine effectively reduced tumor burden and metastatic incidence in a xenograft model of PC. Our mechanistic studies indicate that afatinib acts on PCSC by inhibiting EGFR/ERK/FOXA2 to target SOX9. Altogether, we have shown that afatinib inhibits stemness properties in pancreatic CSCs and in combination with gemcitabine may provide a potent therapy against pancreatic CSCs; thus, this novel combination therapy may improve clinical outcomes for PDAC patients.

## 4.4. Figures and Legends

**Fig.4.1. High self-renewal marker expression in Gemcitabine treated and high-grade pancreatic cancer.**

A) Representative immunofluorescence image of Human tissue microarray with pancreatic cancer and normal pancreatic cores stained with SOX9, CD44, ESA and CD133, CSC and self-renewal markers (PDAC stage II grade 2 malignant, PDAC stage III grade 3 malignant, normal pancreatic tissue). B) Quantitative analysis of CSC marker expression in stained tissue microarray cores. Mean fluorescent intensities for Red, Green and Blue stains was noted for each picture. Red and green staining was then normalized by blue stain to get normalized mean fluorescent intensities. C) Quantification of IHC scoring for expression of SOX9 in patient samples treated with chemotherapy versus no chemotherapy depicting an increase in its expression upon chemotherapeutic treatment. A human tissue microarray with information about the primary and secondary treatments provided to the patients was stained immunohistochemically for expression of SOX9. The array was then scored for SOX9 staining with the help of a pathologist, for comparative analysis cores were divided into two groups; Chemotherapy (the samples that had a history of chemotherapy with gemcitabine of 5FU) and No Chemotherapy (the samples that had no history of chemotherapy). A subset of 7 cores/samples treated with chemotherapy had significantly high expression of SOX9 compared to average staining in no chemo group. These sections were immunohistochemically scored for SOX9 expression.( $P<0.05$: *, $P<0.001$: ** $P<0.0001$: ***)

**Fig. 4.1**

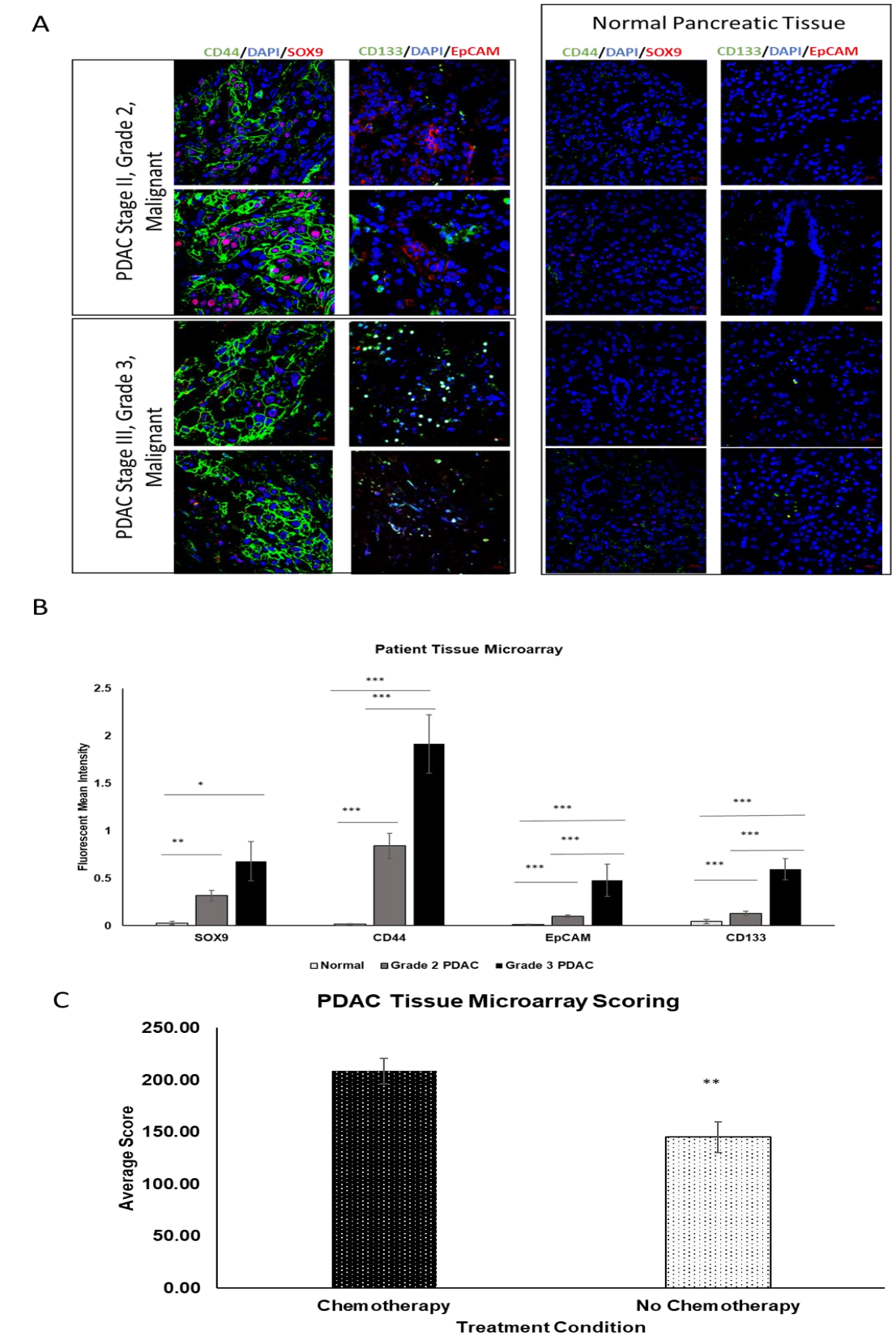

A
Normal Pancreatic Tissue
CD44/DAPI/SOX9
CD133/DAPI/EpCAM
CD44/DAPI/SOX9
CD133/DAPI/EpCAM
PDAC Stage II, Grade 2, Malignant
PDAC Stage III, Grade 3, Malignant
B
Patient Tissue Microarray
Fluorescent Mean Intensity
2.5
2
1.5
1
0.5
0
SOX9
CD44
EpCAM
CD133
Normal
Grade 2 PDAC
Grade 3 PDAC
C
PDAC Tissue Microarray Scoring
Average Score
250.00
200.00
150.00
100.00
50.00
0.00
Chemotherapy
No Chemotherapy
Treatment Condition

**Fig.4.2. Effects of afatinib on the development of pancreatic cancer (PC) organoids**.

A) Experimental scheme is showing the development of organoids and the treatment strategy. B) Microscopic images of hematoxylin and eosin (H&E)-stained KPC mouse PC organoids at days 2, 3 and 5. C) Microscopic images of H&E-stained human PC organoids at days 2, 3 and 5.

Human PDAC organoids were embedded in matrigel domes and seeded in 48 well plates in groups as per the scheme in “4.2A”. After two days in culture all four groups of organoids were observed under microscope and images were clicked. The wells containing these organoids were then marked for the location of these organoids. Drugs were added at concentrations of 0.5µM Afatinib and 0.50.5µM Gemcitabine and control group was given equal microliters of PBS. For the next five days the location and size of these organoids were tracked/followed. Media was changed on day 2 and 4 for both groups with re-administration of drugs and PBS. At the end of five days pictures were clicked again for the tracked organoids. The sizes of tracked organoids were measured by clicking their picture and measuring each organoid using the Moticon Software. Therapy response was evaluated by comparing change in organoids over 5 days in the groups. D) Quantitative analysis of the average size change in control and treated KPC mouse organoid groups (Control, Afatinib, Gemcitabine, Afatinib, and Gemcitabine combination treatments) over 5 days of treatment. E) Microscopic images of KPC PC organoids before and after treatment for 5 days, and H&E-stained sections at the end of the treatment. F) Quantitative analysis of the average size change in control and treated human PC organoid groups (Afatinib, Gemcitabine, Afatinib, and Gemcitabine combination treatments) over 5 days of treatment. G) Microscopic images of human PC organoids before and after treatment for 5 days, and H&E-stained sections at the end of the treatment.

**Fig. 4.2**

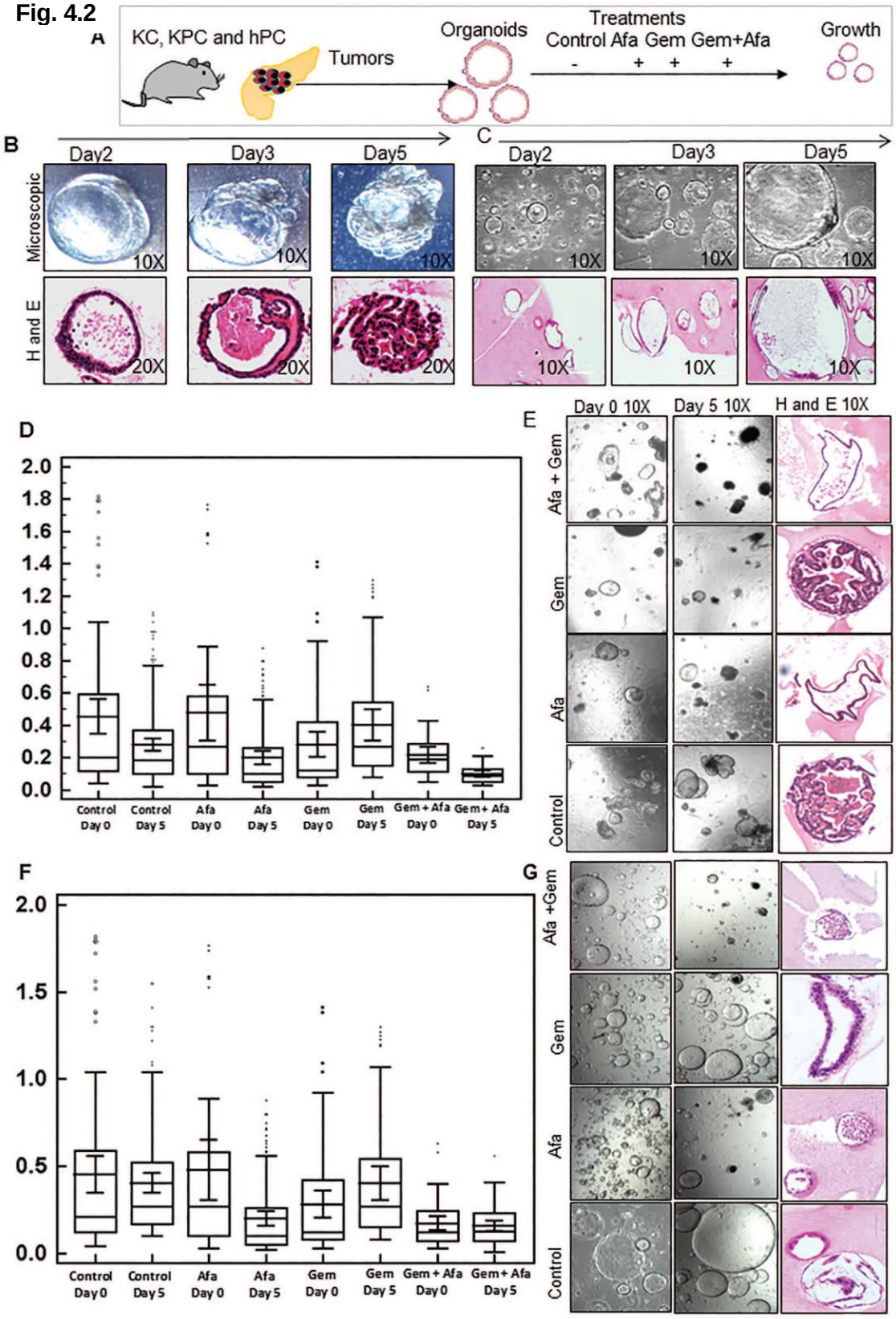

A
KC, KPC and hPC
Tumors
Organoids
Treatments
Control Afa Gem Gem+Afa
- + + +
Growth
B
C
Day2
Day3
Day5
Microscopic
H and E
10X
20X
D
E
Day 0 10X
Day 5 10X
H and E 10X
Afa + Gem
Gem
Afa
Control
Control Day 0
Control Day 5
Afa Day 0
Afa Day 5
Gem Day 0
Gem Day 5
Gem + Afa Day 0
Gem + Afa Day 5
F
G
Afa +Gem

**Fig. 4.3. Afatinib and gemcitabine combination treatment inhibits pancreatic tumor growth and metastasis in an in vivo xenograft mouse model**.

A) Effect of treatments (Afatinib, Gemcitabine alone and Afatinib with Gemcitabine combination treatment) on the EGFR family of proteins in PC cell lines SW1990 and CAPAN1. Cell lines were treated at IC50 concentrations. B) Study design for in vivo mouse experiments. C) Statistical analysis of murine primary tumor weights upon study completion. Upon euthanasia mice tumors were resected and weighed. D) Diffused luminescent imaging tomography (DLIT) images of mice after 4 weeks of treatment before resection. E) Metastasis incidence in mice after 4 weeks of treatment. Upon euthanization internal organs of the mice were harvested and metastatic spots were counted on the organs to calculate metastatic incidence F) Microscopic images of H&E-stained sections of metastasis in vital organs of mice after 4 weeks of treatment.

**Fig. 4.3**

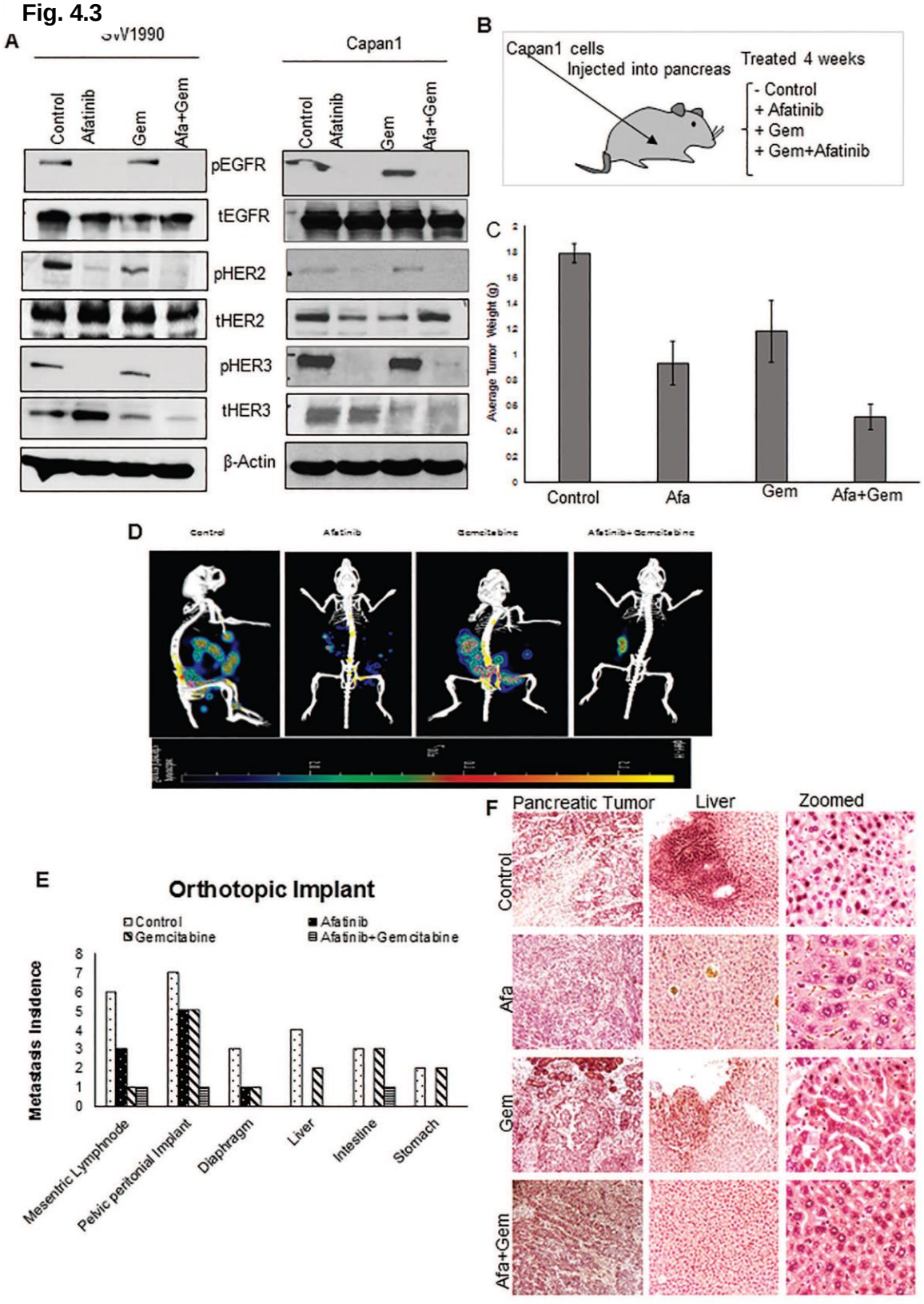

A
SW1990
Capan1
Control
Afatinib
Gem
Afa+Gem
pEGFR
tEGFR
pHER2
tHER2
pHER3
tHER3
β-Actin
B
Capan1 cells
Injected into pancreas
Treated 4 weeks
- Control
+ Afatinib
+ Gem
+ Gem+Afatinib
C
Average Tumor Weight (g)
Control
Afa
Gem
Afa+Gem
D
Control
Afatinib
Gemcitabine
Afatinib+Gemcitabine
E
Orthotopic Implant
Control
Gemcitabine
Afatinib
Afatinib+Gemcitabine
Metastasis Insidence
Mesentric Lymphnode
Pelvic peritonial Implant
Diaphragm
Liver
Intestine
Stomach
F
Pancreatic Tumor
Liver
Zoomed
Control
Afa
Gem
Afa+Gem

**Fig.4.4. Afatinib inhibits cancer stem cell (CSC) counts and stemness.**

A). Experimental design for experiments in the CSC population. B-C). PC cells were seeded and treated with IC50 concentrations of Afatinib, Gemcitabine, a combination of Afatinib and gemcitabine. Following 48 hours of treatment cells were subjected to FACS analysis for side population to identify low Hoechst staining CSC population. Percentage of side population (SP) cells in PC cell lines (B. SW1990 and C. Capan1) after 48 hours of treatment. D-E). FACS analysis showing SP fraction in control and treatment groups (Afatinib, Gemcitabine and Afatinib and Gemcitabine combination treatments) after 48 hours (D. SW1990 and E. Capan1). F-G). Isolated SP/CSC population was seeded in low attachment pates in two groups (Control, Afatinib). IC50 concentration of Afatinib was added to Afatinib after 24 hours of cell seeding. Effects of afatinib on isolated CSC populations in tumor sphere assays; tumor sphere counts, and representative microscopic images are shown (F. SP-SW1990 and G. Capan1).

**Fig. 4.4**

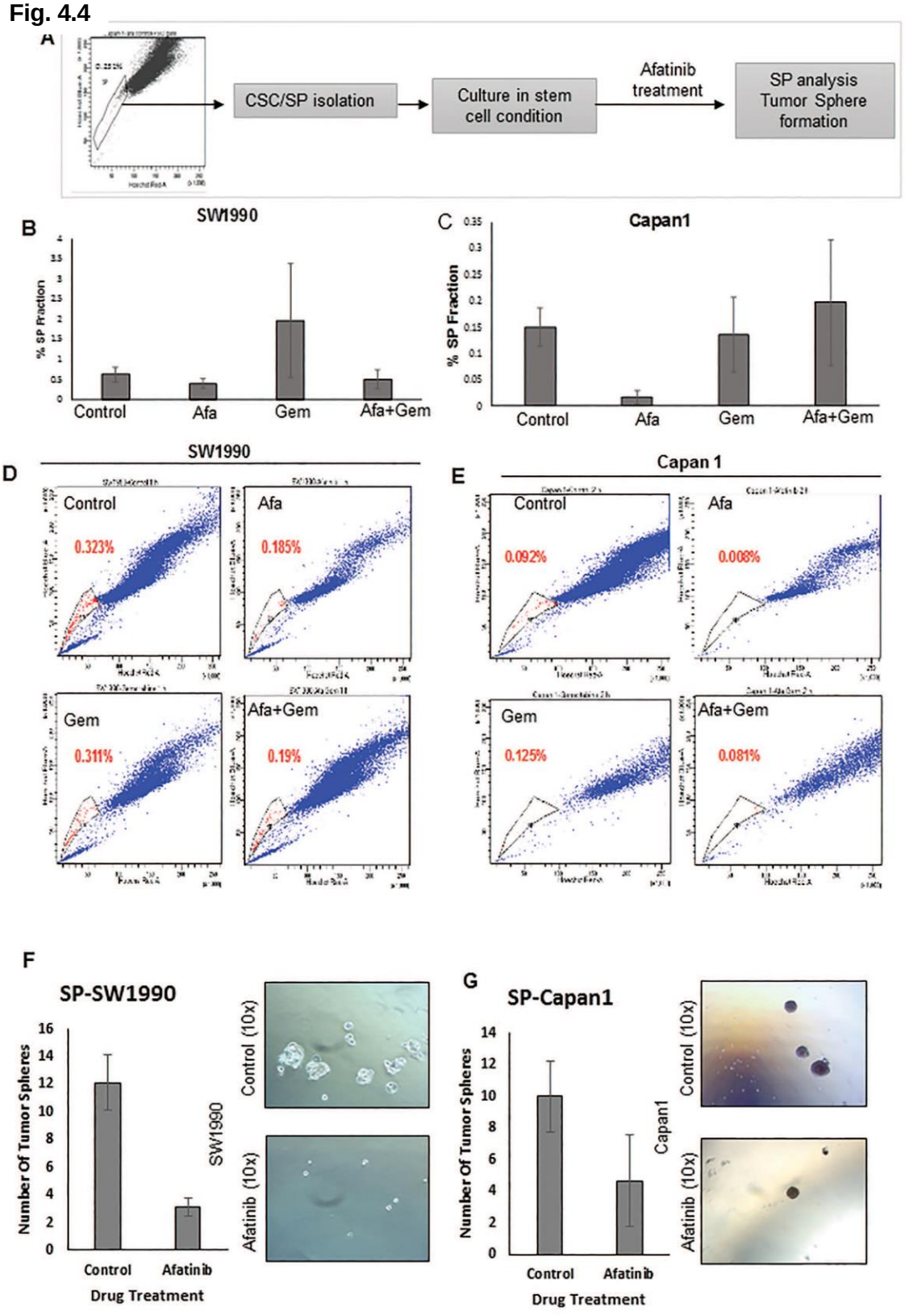

A
CSC/SP isolation
Culture in stem cell condition
Afatinib treatment
SP analysis Tumor Sphere formation
B
SW1990
% SP Fraction
Control
Afa
Gem
Afa+Gem
C
Capan1
% SP Fraction
Control
Afa
Gem
Afa+Gem
D
SW1990
Control
0.323%
Afa
0.185%
Gem
0.311%
Afa+Gem
0.19%
E
Capan 1
Control
0.092%
Afa
0.008%
Gem
0.125%
Afa+Gem
0.081%
F
SP-SW1990
Number Of Tumor Spheres
Control
Afatinib
Drug Treatment
SW1990
Control (10x)
Afatinib (10x)
G
SP-Capan1
Number Of Tumor Spheres
Control
Afatinib
Drug Treatment
Capan1
Control (10x)
Afatinib (10x)

**Fig.4.5. Afatinib and gemcitabine combination inhibits pancreatic CSCs (PCSCs) in KPC mouse tumor organoids and primary mouse tumors.**

A. Immunofluorescence images of CSC markers and pEGFR in KPC mouse organoids after treatment (Control, Afatinib, Gemcitabine and Afatinib with Gemcitabine combination treatments), scale bars=10 μM, zoomed scale bars=5 μM. B. Quantitative analysis of CSC marker expression in stained organoids from panel A. C. Immuno-stained images of CSC markers and pEGFR in mouse primary tumors after treatment (Control, Afatinib, Gemcitabine, and Afatinib and Gemcitabine combination treatments), scale bars=10 μM, zoomed scale bars=5 μM. D. Quantitative analysis of CSC marker expression in stained organoids from panel C.

Fig. 4.5

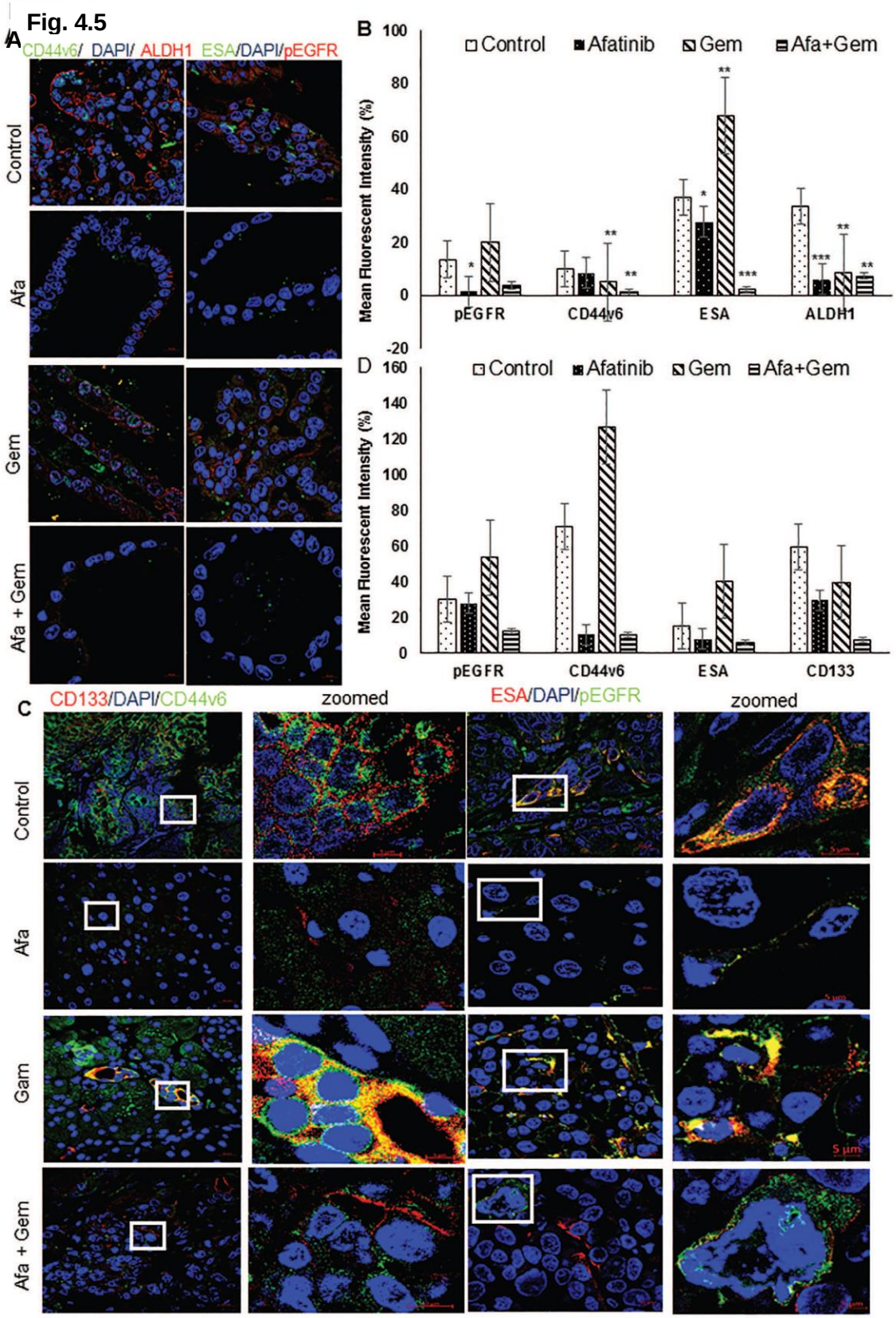

A
CD44v6/ DAPI/ ALDH1 ESA/DAPI/pEGFR
Control
Afa
Gem
Afa + Gem
B
Control
Afatinib
Gem
Afa+Gem
Mean Fluorescent Intensity (%)
pEGFR
CD44v6
ESA
ALDH1
D
Control
Afatinib
Gem
Afa+Gem
Mean Fluorescent Intensity (%)
pEGFR
CD44v6
ESA
CD133
C
CD133/DAPI/CD44v6
zoomed
ESA/DAPI/pEGFR
zoomed
Control
Afa
Gam
Afa + Gem
5 µm

**Fig.4.6. Afatinib inhibits CSC stemness by downregulating multiple CSC markers in PC cells.**

A. Treatment with afatinib downregulates CSC markers and oncogenic markers in PC cells. SW1990 cells were seeded for 24 h and subjected to drug treatment in 4 groups (Control, Afatinib, Gemcitabine and Afatinib, and Gemcitabine combination treatments) at IC50 concentrations for 48 hours, followed by western blot analysis. B. SP cells isolated by Hoechst-based FACS express higher amounts of CSC and self-renewal markers than non-side population (NSP) cells. C. SP-SW1990 cells were treated with IC20 and IC50 concentrations of afatinib, which downregulates the EGFR family of proteins and CSC and self-renewal markers. D-E. Immunofluorescence analysis showing that afatinib represses CSC and self-renewal markers (D. SP-SW1990 and E. SP-Capan1), scale bars=20 μM. F-G. Quantitative analysis of panels D. and E. (F. SP-SW1990 and G. Capan1).

**Fig.4.6**

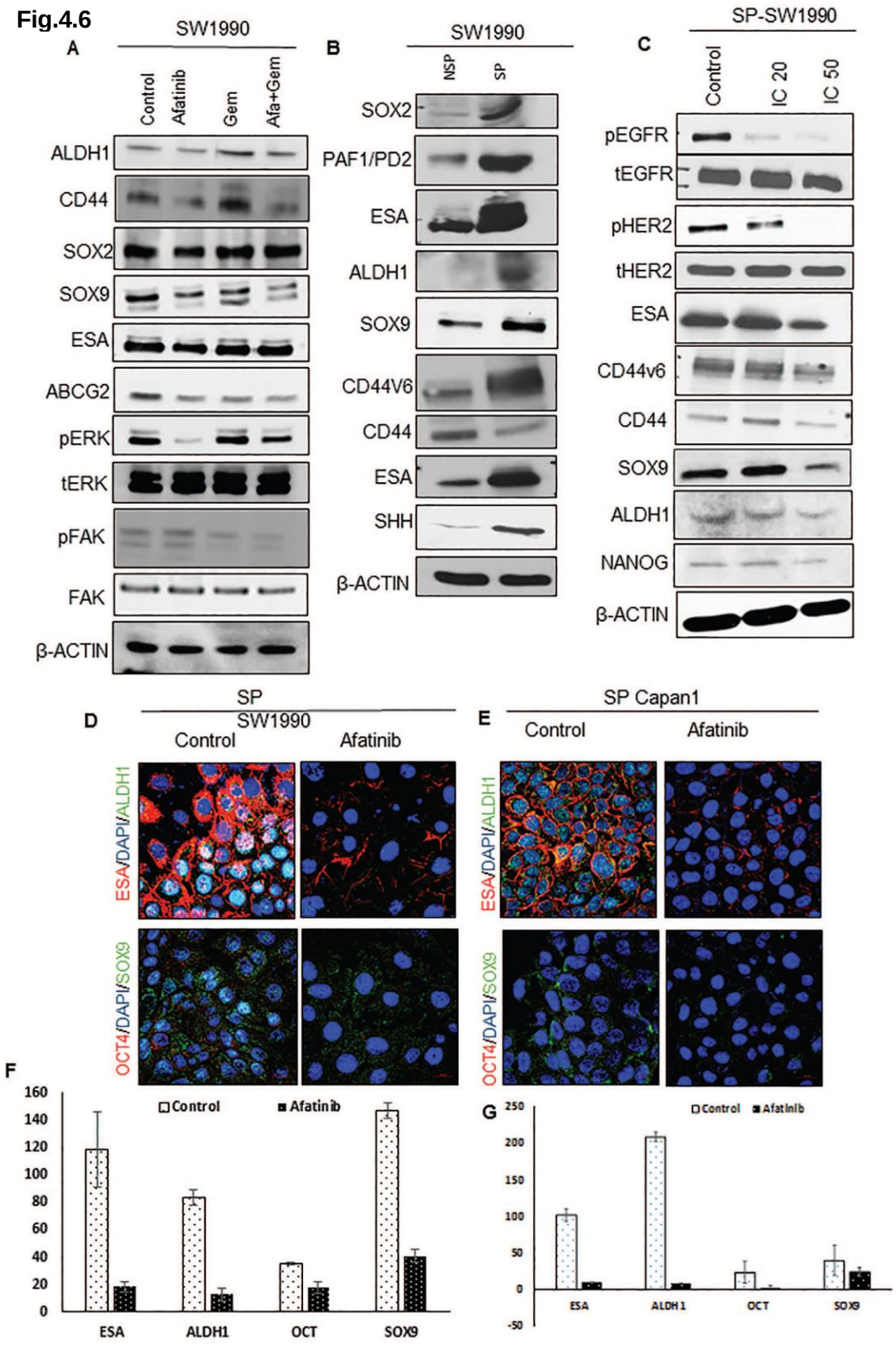
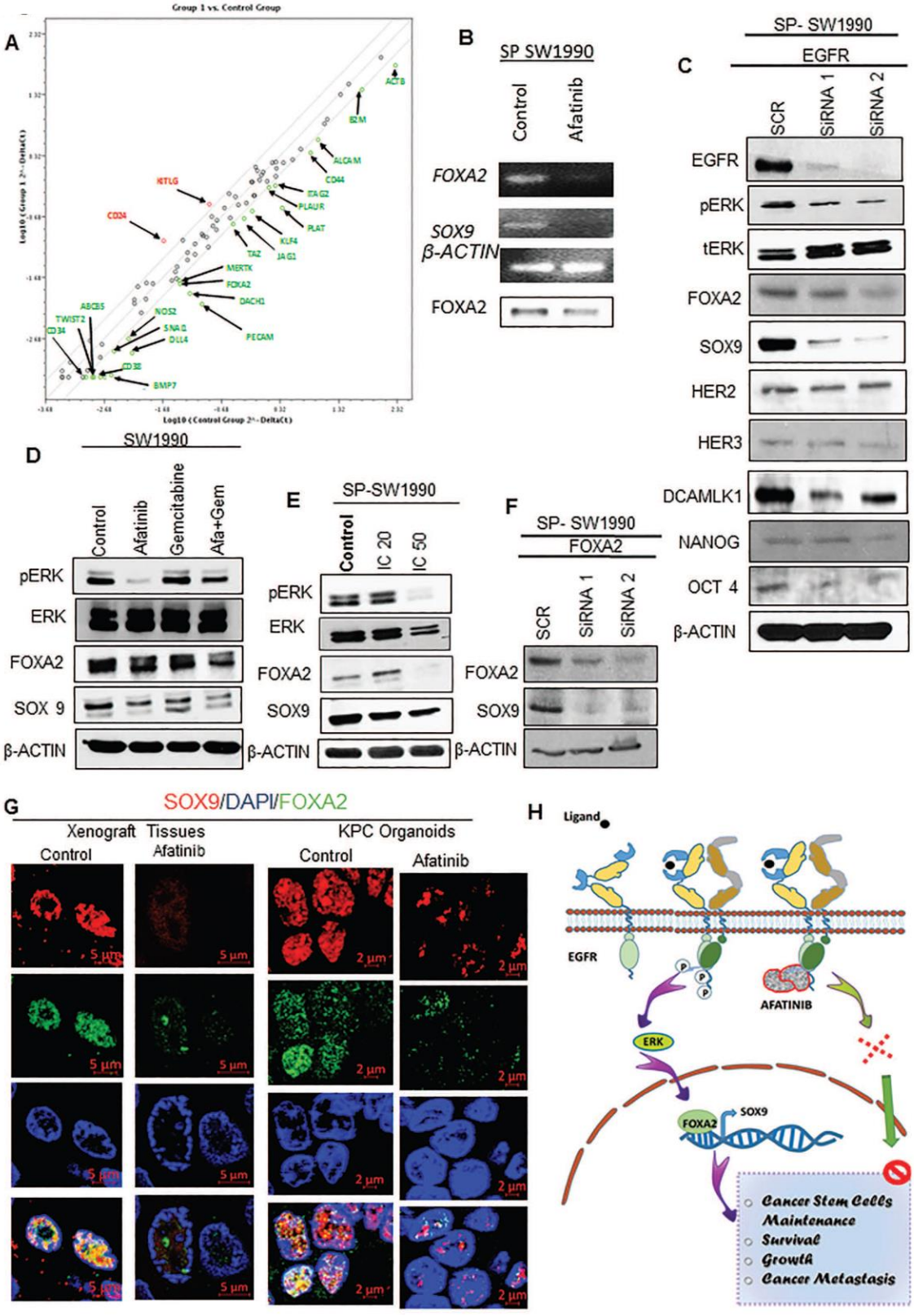

A
SW1990
Control
Afatinib
Gem
Afa+Gem
ALDH1
CD44
SOX2
SOX9
ESA
ABCG2
pERK
tERK
pFAK
FAK
β-ACTIN
B
SW1990
NSP
SP
SOX2
PAF1/PD2
ESA
ALDH1
SOX9
CD44V6
CD44
ESA
SHH
β-ACTIN
C
SP-SW1990
Control
IC 20
IC 50
pEGFR
tEGFR
pHER2
tHER2
ESA
CD44v6
CD44
SOX9
ALDH1
NANOG
β-ACTIN
D
SP
SW1990
Control
Afatinib
ESA/DAPI/ALDH1
OCT4/DAPI/SOX9
E
SP Capan1
Control
Afatinib
ESA/DAPI/ALDH1
OCT4/DAPI/SOX9
F
Control
Afatinib
160
140
120
100
80
60
40
20
0
ESA
ALDH1
OCT
SOX9
G
Control
Afatinib
250
200
150
100
50
0
-50
ESA
ALDH1
OCT
SOX9

**Fig.4.7. Afatinib decreases the CSC population via EGFR/ERK/FOXA2/SOX9 signaling in PCSCs.**

A. PCR array for CSC and self-renewal markers in SP/CSC cells (SP-SW1990) after 4 days of treatment in the culture at IC50 concentrations. B. Afatinib treatment leads to downregulation of FOXA2 and SOX9 RNA and FOXA2 protein. C. Transient repression of EGFR by independent siRNAs in SP-SW1990 cells downregulates pERK, FOXA2, SOX9, DCAMLK1, NANOG, and OCT4. D. Protein expression of pERK, FOXA2, and SOX9 in SW1990 cells upon treatment with afatinib and gemcitabine alone and in combination. E. Downregulation of pERK, FOXA2, and SOX9 at IC20 and IC50 concentrations of afatinib in the SP/CSC population of SW1990. F. Downregulation of SOX9 upon transient knockdown of FOXA2 in the SP/CSC population of SW1990. G. Immunofluorescence images of afatinib-treated KPC tumor organoids and mouse primary tumors depicting the reduction of FOXA2 and SOX9. H. Schematic model of the hypothesized signaling axis by which afatinib inhibits the PCSC, scale bars= 5 μM and 2 μM.

**Fig.4.7**

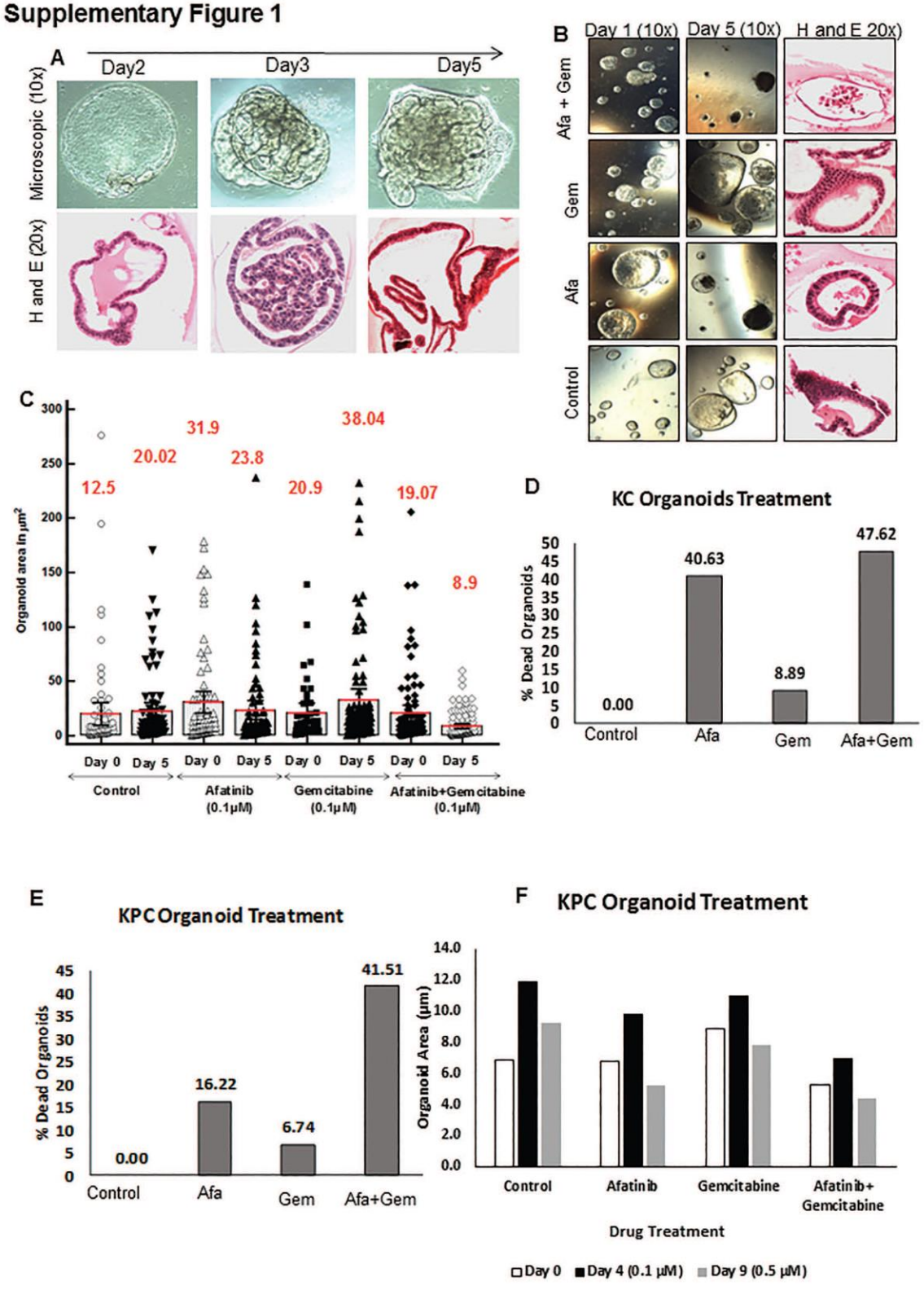

A
Group 1 vs. Control Group
B
SP SW1990
Control
Afatinib
FOXA2
SOX9
β-ACTIN
FOXA2
C
SP- SW1990
EGFR
SCR
SiRNA 1
SiRNA 2
EGFR
pERK
tERK
FOXA2
SOX9
HER2
HER3
DCAMLK1
NANOG
OCT 4
β-ACTIN
D
SW1990
Control
Afatinib
Gemcitabine
Afa+Gem
pERK
ERK
FOXA2
SOX 9
β-ACTIN
E
SP-SW1990
Control
IC 20
IC 50
pERK
ERK
FOXA2
SOX9
β-ACTIN
F
SP- SW1990
FOXA2
SCR
SiRNA 1
SiRNA 2
FOXA2
SOX9
β-ACTIN
G
SOX9/DAPI/FOXA2
Xenograft Tissues
Control
Afatinib
KPC Organoids
Control
Afatinib
5 µm
2 µm
H
Ligand
EGFR
AFATINIB
ERK
FOXA2
SOX9
Cancer Stem Cells Maintenance
Survival
Growth
Cancer Metastasis

## Supplementary Figures

### Supplementary Figure 1.

Effects of afatinib on KC and KPC organoids. A. Microscopic images of H&E-stained PC organoids from KC mice. B. Microscopic images of KC mouse organoids before and 5 days after treatment, and H&E-stained sections at the end of the treatment. C. Quantitative analysis of the average size change in control and treated KC PC organoid groups (Afatinib, Gemcitabine, and Afatinib and Gemcitabine combination treatments) over 5 days of treatment. D-E. Percentage of organoids estimated to be dead based on microscopic analysis of the morphology. D. KC tumor organoids. E. KPC tumor organoids. F. Quantitative analysis of the average size change in control and treated KPC mouse organoid groups (Afatinib, Gemcitabine and Afatinib and Gemcitabine combination treatments) at days 4 and 9, indicating desired concentration for treatment

**Supplementary Figure 1**



**Supplementary Figure 2.**

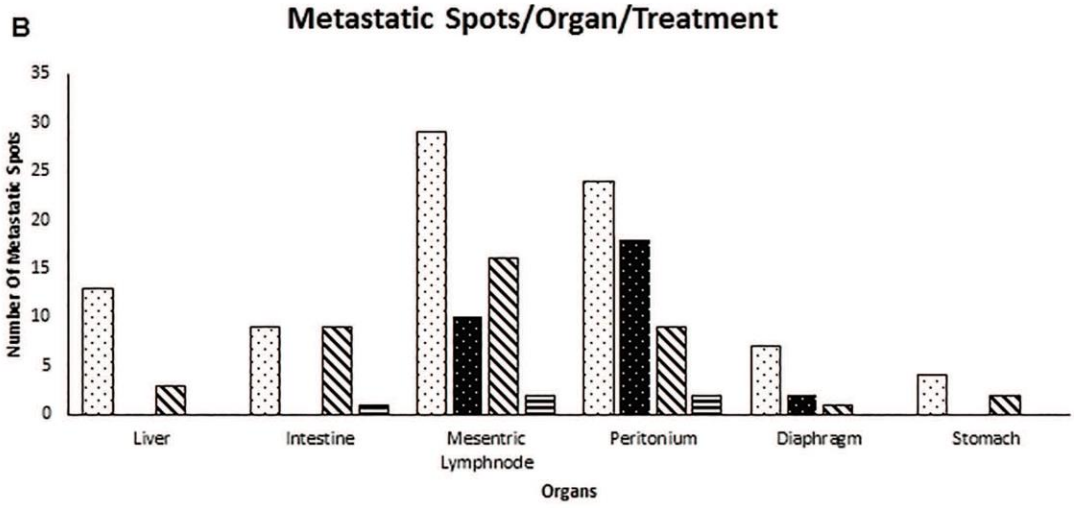
A). To judge the sensitivity of PC cells (SW1990 and Capan1) to gemcitabine and afatinib, the IC50s for both drugs were calculated for PC cell lines and their respective SP/CSC isolated line. B) Mice were orthotopically injected with Capan1 cells and treated in 4 groups (control, Afatinib, gemcitabine, a combination of gemcitabine and Afatinib) after 4 weeks of implantation. After 3 weeks of treatment, mice were euthanized, and internal organs of the mice were harvested, a number of metastatic spots were counted on each organ. Histogram depicting the number of metastatic spots per organ. C) Dot plot depicting the correlation between tumor size and metastasis. Metastasis incidence and primary tumor weights were calculated for the mice and to evaluate the impact of tumor size on metastasis the correlation between the two was calculated. The figure depicts a lack of correlation between primary tumor size and metastasis incidence suggesting the decrease in metastasis could have been due to the therapy and not the primary tumor size.

## Supplementary Figure 2

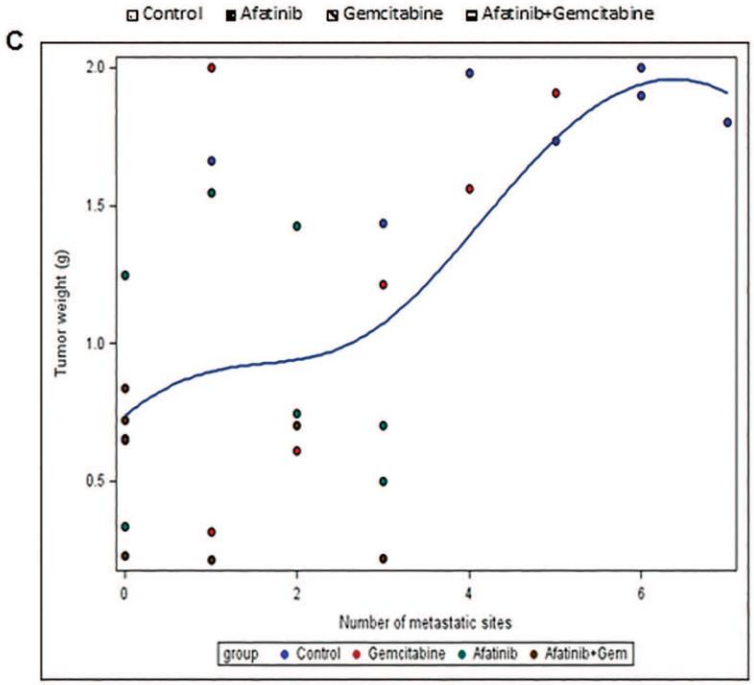
A

| IC50 | AFATINIB (μM) | GEMCITABINE (μM) |
|---|---|---|
| SW1990 | 0.1788791 | 3.702953586 |
| SP SW1990 | 0.069187136 | 4.684968 |
| SP Capan1 | 0.26 | 7.94 |
| Capan1 | 0.49312 | 2.12114 |

B



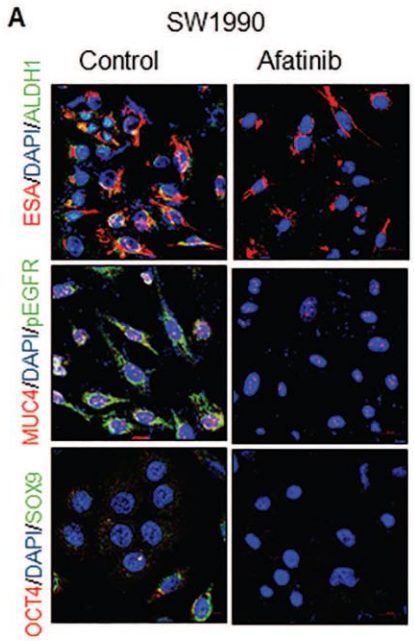

C

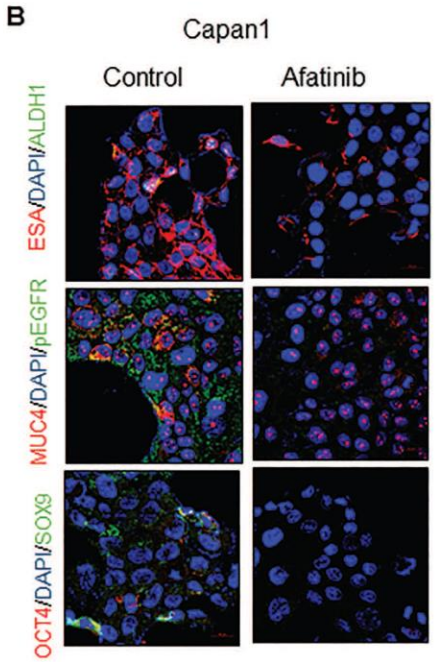


**Supplementary Figure 3.**

Immunofluorescence images of SW1990 and Capan1 cells, showing downregulation of self-renewal and oncogenic markers, A. SW1990, B. Capan-1. Scale bars=20μM.

**Supplementary Fig 3**

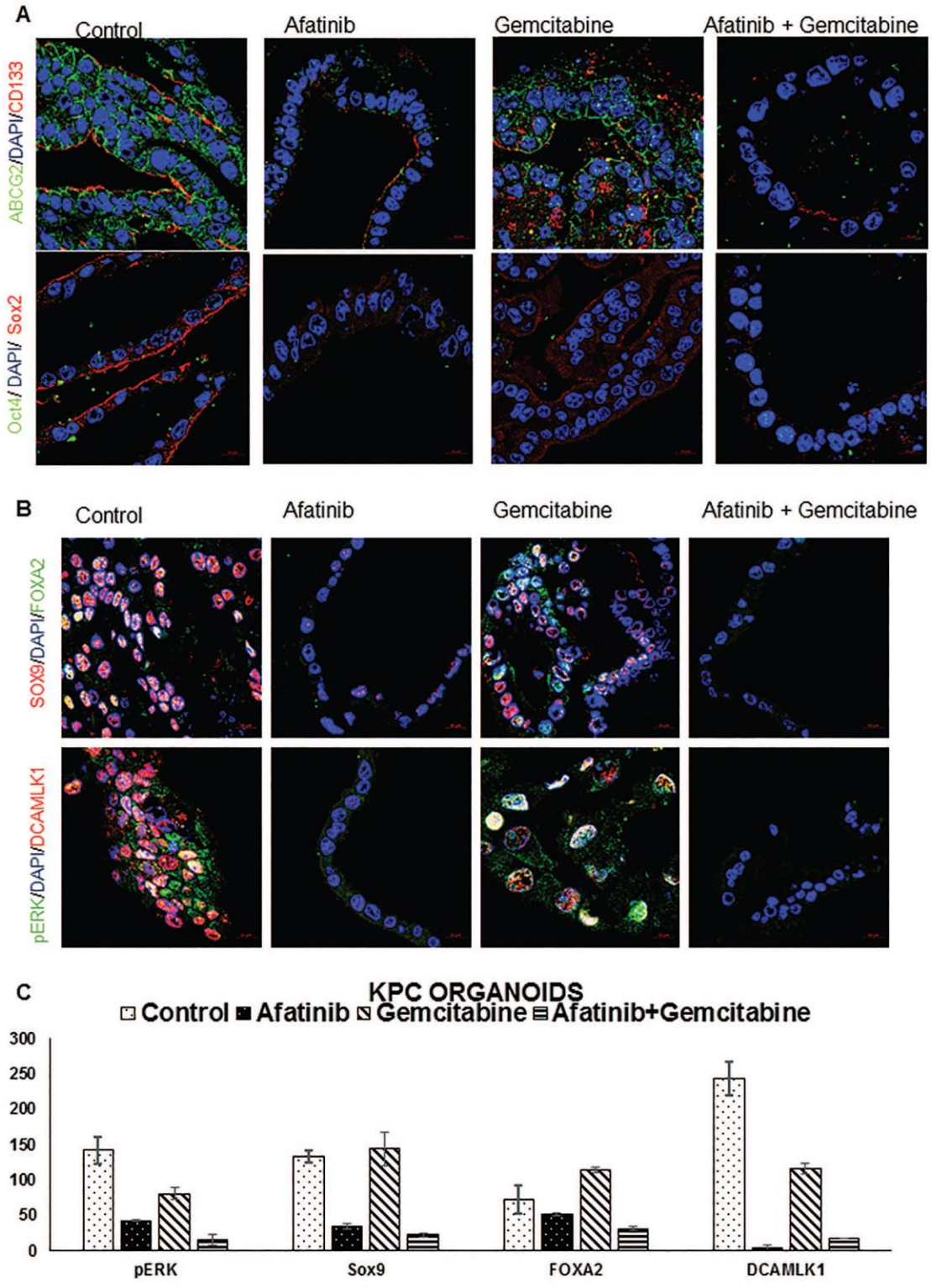


**Supplementary Figure 4.**

A. Downregulation of other CSC and self-renewal markers upon treatment (Control, Afatinib, Gemcitabine, and Afatinib and Gemcitabine combination treatments) in KPC organoids, Scale bars=10 μM. B. Immunofluorescence images of KPC organoids treated with afatinib, depicting a reduction in pERK, FOXA2, and SOX9 along with CSC marker DCAMLK1, scale bars=10 μM. C. Quantitative analysis of CSC and self-renewal markers of panel B.

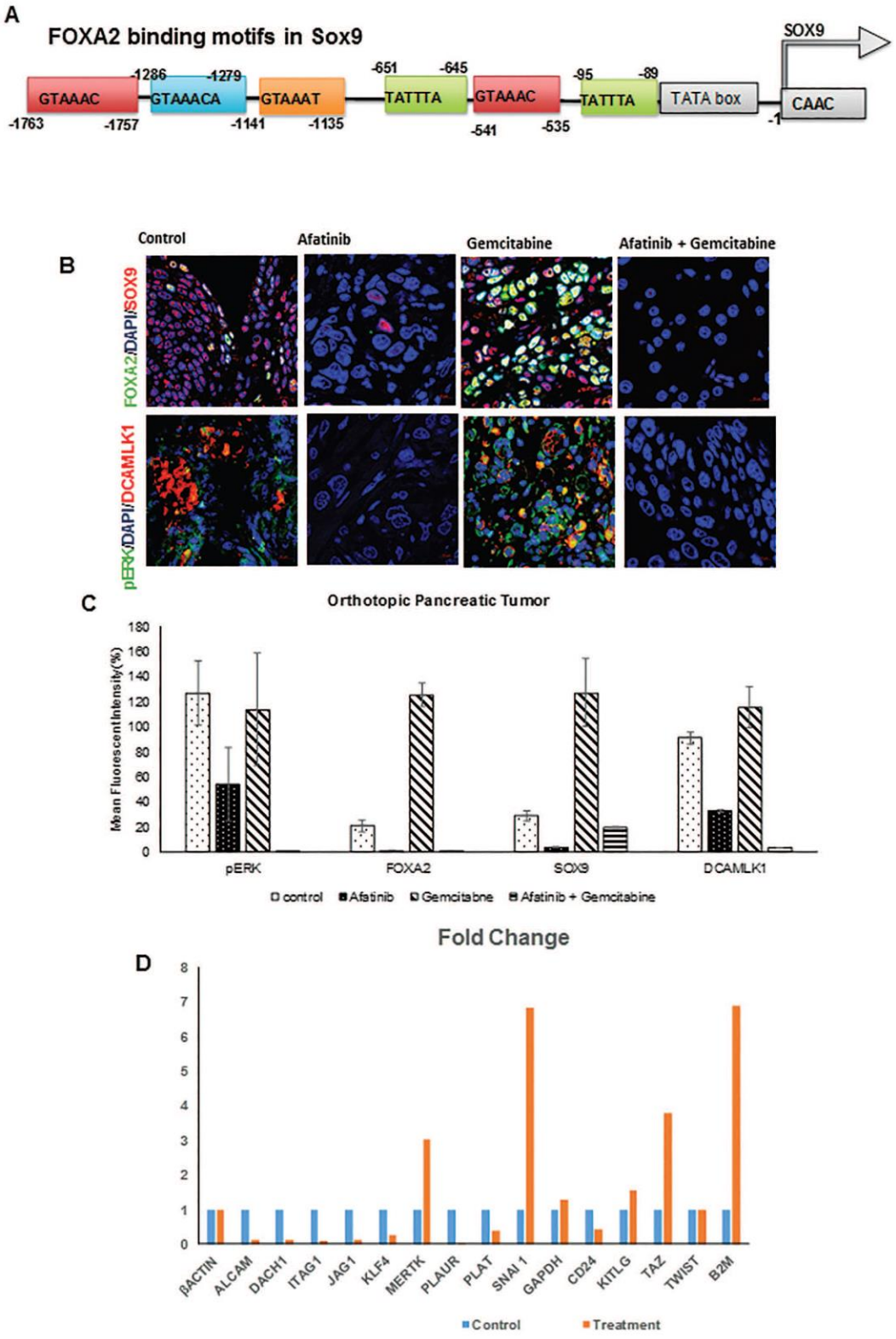
**Supplementary Fig. 4**



**Supplementary Figure 5**.

A. FOXA2 binding motifs for SOX9 promoter region. B.Immunofluorescence images of KPC mouse primary tumors after 4 weeks of afatinib treatment, depicting a reduction in pERK, FOXA2, and SOX9 along with CSC marker DCAMLK1, scale bars=10 μM. C. Quantitative analysis of panel B. D. qPCR analysis of CSC and self-renewal markers found reduced in CSC PCR array from figure 6A.

## Supplementary Fig 5



# CHAPTER 5: SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

## 5.1 Summary

### 5.1.1 Organoids for preclinical research

Three-dimensional (3D) cultures use the inherent nature of cells to self-organize and generate structures that can be programmed to represent an organ or a pathology. Organoid cultures are the 3D cultivation of source tissue (ranging from cells to tissue fragments) in a support matrix and specialized media that nearly resembles the physiological environment. Depending on the source tissue, growth factors, and inhibitors provided, organoids can be programmed to recapitulate the biology of a system and progression of pathology. Organoids are genetically and phenotypically stable, and genetically amenable, making them very suitable tools to study tissue homeostasis and cancer.

One of the goals of the dissertation was to develop in vitro three-dimensional models to test therapeutic response. For this, we developed pancreatic cancer organoids from KC ($Kras^{G12D}$; $Pdx1^{Cre)}$, KPC ( $Kras^{G12D}$; $Trp53^{R172H/+}$; $Pdx1^{Cre}$ ) and PDAC patient samples. This was achieved by modifying protocol from the pioneering Hutch et al. publication and further standardizing it for our laboratory and experimental needs. Our organoids grew in culture and demonstrated a change in morphology, from being organized single cellular ductal structures to unorganized structures. Our tumor organoids mimic PDAC architecture and environment *in vitro*, although with a lack of immune environment. These could be later used as models to study PDAC and drug response studies. We also report the development of lung organoids and prostate organoids for future utilization.

### 5.1.2 Afatinib targets CSCs in PDAC

PC is a highly lethal disease, and CSCs are major culprits for the aggressive and metastatic nature of this disease. Present therapies act on differentiated cancer cells and fail to target CSC. EGFR family of proteins is important for CSC and PC. Afatinib is an FDA-approved pan-EGFR inhibitor and demonstrated superiority *in vitro* over erlotinib, a currently used drug for PC. Here we used traditional monolayer cultures, 3-D organoid cultures and *in vivo* animal models to show that inhibition of EGFR family of proteins using afatinib attenuates PCSC by specifically targeting a novel EGFR/ ERK/ FOXA2/ SOX9 axis and reduces PC growth and metastasis. This study highlights the importance of targeting CSCs and shows the therapeutic potential of the combination of afatinib and gemcitabine.

In this study, the primary goal of my dissertation was to develop a targeted therapy inhibiting cancer stem cells against pancreatic cancer. For which we decided to focus on targeting EGFR since it is vital for cancer stem cell maintenance and highly expressed in PC as well as pancreatic cancer stem cells. We have used Afatinib out of several EGFR family inhibitors. Inhibitory effects of Afatinib were revealed when a significant reduction in PSCS population was observed after Afatinib treatment. Additionally, Afatinib inhibited tumorigenic, and self-renewal potential of PCSCs and down-regulated several vital PCSC markers and self-renewal markers in PC cells as well as the PCSCs. These results suggested a targeted action of Afatinib on PCSCs, which are otherwise resistant to most chemotherapeutic drugs including gemcitabine. We demonstrated in several experiments that gemcitabine treatment, which a very widely used chemotherapeutic drug against PC, leads to an enrichment of PCSCs when given alone whereas in combination with Afatinib treatment. This was revealed when after gemcitabine treatment an increase in PCSC population and enhanced expression of

CSC markers and self -renewal markers in PC cells but not in combination treatments. We demonstrated that in an orthotopic mouse model of PC Afatinib treatment inhibited PC and metastasis, and a combination of Afatinib and gemcitabine showed a better inhibition when compared to both the drugs alone. We could successfully achieve our first objective and inhibit PDAC and target PCSCs.

CSCs are known to be major culprits for PDAC metastasis, and hence we expected that Afatinib treatment alone and in combination would inhibit CSC markers like CD133, CD44, and SOX9 in orthotopic mouse models tumor sections. Which we could test and demonstrate by Immunofluorescence staining of these tumor sections. We also tested the proposed therapy in 3-D tumor organoid model, since they are known to maintain a robust population of CSCs, and demonstrated a similar reduction in CSC markers and self-renewal markers. Our PCR Microarray analysis of CSC makers and self renewal markers revealed a list of 23(*ABCB5, ALCAM, BMP7, CD38, CD44, DACH1, DLL4, FOXA2, ITGA2, JAG1, KLF4, MERTK, NOS2, PECAM1, PLAT, PLAUR, PTPRC, SNAI1, TAZ, TWIST2, ACTB, B2M and HGDC*) makers which were down-regulated. Amongst them was FOXA2 which is known to be regulated by and regulate SOX9 a master regulator for several CSC maintenance genes. FOXA2 is also regulated by pERK which is a downstream molecule in EGFR signaling. For further investigation, if this mechanism is being followed, we knocked down EGFR using 2 independent siRNAs and revealed a downstream downregulation of pERK, FOXA2and SOX 9 along with other CSC markers. We also knocked down FOXA2 in a similar fashion and observed a down-regulation of SOX9. We could identify a possible mechanism of action of Afatinib through EGFR/ERK/FOXA2/SOX9 axis. Immunofluorescent staining of tumor organoids treated with Afatinib and tumor sections from an orthotopic mouse model of PDAC showed a similar down-regulation of FOXA2 and SOX9 and hence confirmed or

mechanism of action in the 3D model and in vivo environments. Overall, we demonstrated a combination of Afatinib and gemcitabine could provide a potential targeted therapy for PDAC and its metastasis. My next goal is to understand how Afatinib and EGFR inhibition modifies the tumor microenvironment in PDAC. We are currently in the process of investigating the effect of Afatinib on CAFs and developing a 3-D model to provide a more holistic picture of the therapy.

Overall, our studies provide a targeted therapy against PCSCs using Afatinib and gemcitabine against PDAC and its metastasis. This study will also prove that cytotoxic therapies, like Gemcitabine, do not target pancreatic CSC, may be detrimental for patients. On the other hand, Afatinib, which has a specific action on pancreatic CSC along with Gemcitabine, would yield much better patient survival.

## 5.2 Conclusions

Our overall objective was to generate targeted therapy against pancreatic cancer that inhibits cancer stem cells as well. We generated organoid cultures to facilitate drug response studies since 3D organoids provide a more robust platform that simulated disease architecture, biology and in most part heterogeneity. Since these aspects are vital to generating translatable therapies and are not recapitulated by traditional culture methods first part of this thesis holds much significance. We generated organoids from wildtype murine pancreas, KC, KPC mouse pancreatic cancer models and human patient samples for immediate use in this study and lung and prostate organoids for future metastatic studies.

In the second part of my thesis, we evaluate a combination therapy of Afatinib and gemcitabine against PDAC, its metastasis and cancer stem cell population in PDAC. We report that Afatinib downregulates stemness properties of pancreatic cancer stem cells by downregulating EGFR signaling through ERK. The downregulation of phosphorylated form of ERK decreases FOXA2 expression. Since FOXA2 and SOX9 regulate each other's expression, a decreased expression of FOXA2 causes a decrease in SOX9 which results in downregulation of several self-renewal genes and CSC markers. Due to the inhibitory action of Afatinib an PCSCs it cases a reduction in metastasis incidence as well as potentiates gemcitabine therapy. Hence, we report a combination therapy of Afatinib and gemcitabine can inhibit PCSCs and reduce metastasis as well as inhibit PDAC.

## 5.3. Future Directions

### 5.3.1 Organoids for preclinical research

We successfully generated murine organoids from KC, KPC pancreatic tumors and wildtype pancreas. We also report the generation of organoids from patient pancreatic tumors and adjacent normal pancreatic tissue.

Recent findings have reported preservation of histopathological features of the original tumors both in vivo and following xenotransplantation studies in immune-deficient mice [227]. This suggests that in vitro drug responses can be examined in vivo in a more complex environment by organoid transplantation. The generated PDAC organoids can be transplanted in the immune deficient mouse to further validate the therapy response.

A tumor organoid biobank was generated from 20 CRC patients and their matching normal tissue [228]. Integration of genomic and monotherapy drug response data revealed several therapeutically exploitable relations. One of the organoid cultures from the study was found to be sensitive to inhibition of WNT secretion due to the presence of RNF43 (WNT antagonist E3 ubiquitin ligase) [147, 229] . Several already known correlations between genetic mutations and drug responses were also observed along with differential cytotoxicity profiles with no genetic predictive marker. These results warrant further validation and hence highlight the need for the development of more biobanks. Last year a study reported the genetic characterization of organoid biobank from 39 PDAC patients [184]. The study revealed three distinct PDAC organoid subtypes based on gene expression subtypes revealing distinct WNT signaling dependencies. There is a need for the generation of more PDAC organoid biobanks for the generation of statistically significant inferences about therapy response and toxicology studies [230].

Another recent study reported generation of PDAC Organoid lines from 17 patients [176]. Organoids were reported to follow parental tumor histological features both in vitro and upon xenotransplantation although mutation analysis was not performed on the organoids. The study reveals incapability of generation of organoid from poorly differentiated to moderately differentiated PDACs suggesting a modification of protocol to allow more conclusive therapy response studies. The cell of origin of PDAC has been a controversial subject. Organoids generated so far have mostly been of ductal origin, generation of acinar organoids is critical to study the prospective role of ADM in PDAC (Acinar to ductal metaplasia). A study utilized a genetically engineered inducible mouse model of ADM to generate organoids that undergo ADM in vitro[231]. The study revealed that both acinar and ductal cells of origin require identical oncogenic drivers to trigger PDAC but differ in pathophysiology and marker expression. There is a need for developing protocols to generate acinar cell organoids from patient samples to allow further studies into studies of PDAC origin.

Patient-derived organoids were generated from metastatic gastrointestinal cancer in a recent study and used to predict patient therapy response ([232]). Authors tested a compound library of drugs used either in current clinical trials or clinics. A positive predictive value of 88% (predicting that a particular drug works) and a negative predictive value of 100% (predicting that a particular drug does not work) was revealed in the study suggesting recapitulation of therapy responses from clinical trials. The study highlights the possibility of the development of personalized medicine platforms[232].

Studies have reported genetic modeling of cancer from normal organoids in CRC as well as PDAC [176, 233]. Co-culture systems have been established that allow expansion of hematopoietic cells in vitro with organoids. Short-term maintenance of intraepithelial lymphocytes has also been achieved in co-culture with intestinal organoids upon supplementation with interleukin-2 (IL-2), IL-7 and IL-15 (Nozaki et al. 2016).

Another recent study demonstrated maintenance of CD45+ lymphocytes up to 8 days in the air-liquid interphase organoid system [234]. These studies highlight the role of organoid co-culture systems for future studies. We have developed normal lung and prostrate organoids along with pancreatic cancer organoids. A co-culture system can be generated that includes normal organoids co-cultures with cancer organoids to simulate pre-metastatic niche priming and metastasis in PDAC.

### 5.3.2. Afatinib targets CSCs in PDAC

Our studies have convincingly demonstrated that Afatinib inhibits pancreatic cancer stem cells via EGFR/ERK/SOX9 axis and in a combination of gemcitabine inhibits pancreatic cancer and its metastasis.

The interaction between the tumor and its microenvironment is complex, and a lot is still unknown about the communications between tumor cells and tumor microenvironment. Hypoxia and immune suppression are major hallmarks of the tumor microenvironment. The programmed cell death-ligand 1 (PD-L1) on tumor cells is a major part of the immune suppression module [235]. PD-L1 interacts with programmed cell death-1 (PD-1) on immune cells in the tumor microenvironment to avoid host immune surveillance [236]. Tumor cell or normal cell secreted cytokines can enhance tumor growth, increase metastasis and angiogenesis along with enabling immune evasion [237, 238]. Several studies have examined inhibition of PD-1/PD-L1 immune checkpoint signaling to target immune evasiveness of tumors. Expression of PD-L1 has also been proposed as a predictive marker for checkpoint inhibition therapy [239]. A correlation has been observed in the expression of PD-L1 in tumors with better prognosis with EGFR tyrosine kinase inhibitors in NSCLCs ([240]). Better EGFR TKI treatment outcome has been observed in EGFR mutant NCLCs patients, and EGFR mutation status is positively associated with high PDL1 expression [241, 242]. Enhanced

infiltrations by TILs (tumor-infiltrating lymphocytes) is associated with decreased metastatic recurrence in HER2-positive breast cancer treated with the anti-HER2 monoclonal antibody, trastuzumab [243]. These studies suggest an association between EGFR/HER2 status and immune evasion and hence, it will be interesting to observe the effects of Afatinib on PDAC immune evasion.

Another study has suggested the role of tumor microenvironment in the acquisition of resistance to Afatinib treatment in NCLCs through HGF secretion [243]. Several mechanisms have been reported for acquired resistance to Afatinib treatment although functional studies are needed to prove a causative relationship with resistance and most of them have been elucidated in lung cancer. Some of these resistance mechanisms include V843I EGFR mutation and c-MET amplification, enhanced IL6R/JAK/STAT signaling and expression of FGFR1. Additionally, increased interference with aerobic glycolysis and autophagy are also proposed to be associated with Afatinib resistance [228]. A recent study reported acquisition of resistance to EGFR family inhibitors by activation of STAT3 and simultaneous upregulation of p-c-MET, p-STAT3, CD44, increased autocrine production of EGFR ligand amphiregulin and differential activation status of EGFR tyrosine residues as well as downregulation of total and p-SRC [190]. The results were majorly cell line based, and further studies are needed to elucidate the mechanism behind Afatinib resistance.

Afatinib is currently under clinical trials examining its usage as neoadjuvant therapy. Considering its role against cancer stem cells unlike chemotherapies administering Afatinib at an early stage may be an effective therapeutic regimen. The recent release of interim results from phase II ASCENT trial in lung cancer are encouraging for future studies in this direction. The trial examines Afatinib as the standard of care treatment with curative intent for EGFR+ stage III NSCLC

(NCT01553942). Neo-adjuvant afatinib achieved a high objective response rate and major surgical path responses.

Additionally, the results also suggest that neoadjuvant Afatinib exceeds feasibility over adjuvant Afatinib in stage III patients. An enhanced progression-free survival has also been observed although; more data is needed about optimal strategy for stage III EGFR+ pts [244]. These studies provide a case for testing Afatinib in the neoadjuvant setting in PDAC along with genotype-based patient stratification.

# CHAPTER 6: REFERENCES

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