
The Impact of Clonal Heterogeneity and Plasticity on Malignancy Associated Properties and Therapy Response in Pancreatic Cancer

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„Leicht ist das Leben für keinen von uns. Doch was nützt das, man muss Ausdauer haben und vor allem Zutrauen zu sich selbst. Man muss daran glauben, für eine bestimmte Sache begabt zu sein, und diese Sache muss man erreichen, koste es, was es wolle.“

Marie Curie (1867 – 1934)

Zusammenfassung

Das duktales Pankreasadenokarzinom (engl.: *pancreatic ductal adenocarcinoma*; PDAC) ist die häufigste, maligne Erkrankung des Pankreas. Aufgrund der spät auftretenden, unspezifischen Symptome wird das PDAC meist erst in einem weit fortgeschrittenen oder bereits metastasierten Stadium diagnostiziert, was es zu einer der tödlichsten Tumorentitäten macht. Die einzige Chance auf Heilung stellt eine operative Resektion des Tumors dar, welche jedoch aufgrund der häufig späten Diagnose nur für etwa 20% der Patient(inn)en in Betracht kommt. Selbst die Mehrheit der Betroffenen, bei denen der Tumor reseziert werden konnte, entwickeln innerhalb weniger Monate bis weniger Jahre lokale Rezidive oder Metastasen. Auch die sich schnell ausbildenden Resistenzen gegenüber den gängigen Chemotherapeutika erschweren die Behandlung. Daher ist die Forschung nach der Ursache für das aggressive Metastasierungsverhalten und das schlechte Therapieansprechen des PDAC von großer Bedeutung.

Krebsstammzellen (engl.: *Cancer stem cells*; CSCs) haben die Eigenschaft zur Selbsterneuerung, unlimitierter Proliferation und sind in der Lage, unterschiedliche Krebszellphänotypen, so auch differenzierte Krebszellen, hervorzubringen. Des Weiteren zeichnen sich CSCs durch ihre Resistenz gegenüber Chemotherapien aus. Diese Versatilität der CSCs steht unter anderem im Zusammenhang mit dem Prozess der Epithelial-Mesenchymalen-Transition (EMT) und dem reversen Prozess der Mesenchymal-Epithelialen-Transition (MET), bei dem epitheliale Zellen mit starkem Proliferationspotential ihren Phänotyp alternieren und mesenchymale Charakteristika mit hohem Invasionspotential annehmen und umgekehrt. Bislang ist noch wenig bekannt, ob CSC-Eigenschaften immer an die EMT/MET gekoppelt sind, oder ob es verschiedene CSC-Phänotypen gibt, die sich auch funktionell unterscheiden. Das Ziel der vorliegenden Studie war es daher, unterschiedliche PDAC-Zelllinien hinsichtlich ihres CSC- und EMT-Phänotyps zu charakterisieren und dessen Auswirkung auf das Ansprechen unterschiedlicher Therapien zu untersuchen. Aus PDAC-Zellen unterschiedlichen Ursprungs wurden CSC-Klone und nicht-CSC Klone isoliert und vergleichend zu ihrer parentalen Zelllinie untersucht. Für diesen Zweck wurden Panc1 Zellen aus einem primären PDAC und Panc89 Zellen aus einer Lymphknotenmetastase verwendet. Die Charakterisierung ergab, dass epitheliale und mesenchymale Eigenschaften an unterschiedliche Stammzellfaktoren gekoppelt sind und dass eine hohe Heterogenität sowohl zwischen parentalen Panc1 und Panc89 Zellen als auch zwischen den generierten CSC und nicht-CSC Populationen besteht. Panc1 CSCs wiesen hohe Expressionen der mesenchymalen Marker L1CAM und Vimentin, des EMT-Induktors ZEB2 sowie des

CSC-Markers Nestin auf. Panc89 CSCs hingegen zeigten eine hohe Expression des epithelialen Markers E-cadherin und des CSC-Markers SOX2. Das Expressionsmuster des Panc89 nicht-CSC Klons wies auf einen partiellen/hybriden EMT-Phänotyp hin. Für diesen Zellklon konnte sowohl die Expression von E-cadherin, L1CAM und Vimentin als auch von dem EMT-assoziierten CSC-Marker OVOL2 nachgewiesen werden. Funktionelle Analysen ergaben eine gesteigerte Wachstumsrate von allen Panc89 Zelllinien verglichen mit allen Panc1 Zelllinien, jedoch mit einer höheren Wachstumsrate der parentalen Zellen verglichen mit den isolierten Zellklonen. Eine Ausnahme bildete hier der Panc89 CSC- Klon, welcher die gleiche Wachstumsrate aufwies wie die parentalen Zellen. Die Untersuchung des Metastasierungspotentials *in vivo* ergab, dass durch Panc89 Zellen verursachte Tumore größer und primär im Peritoneum lokalisiert waren, im Vergleich zu den von Panc1 Zellen gebildeten Tumoren, welche kleiner waren und vorrangig im Pankreas detektiert wurden. Des Weiteren konnte festgestellt werden, dass die Anzahl an gebildeten CSC-Tumoren höher war als die Anzahl der entsprechenden Tumore der nicht-CSC Populationen. Hinsichtlich des Überlebens zeigte sich ein deutlich kürzeres medianes Überleben bei Tieren denen Panc89 CSC und nicht-CSC Populationen injiziert wurden, verglichen mit Panc1 CSC und nicht-CSC injizierten Tieren, die länger überlebten. Hinsichtlich des Therapieansprechens wurden evolutionsbiologische Therapiestrategien mit wechselnden Medikamenten und in Präsenz oder Absenz von Behandlungspausen durchgeführt. Es wurde festgestellt, dass eine durchgeführte Behandlungspause zwischen zwei Wirkstoffen lediglich bei Panc1 CSCs zu einem gesteigerten Therapieansprechen führte, während die Behandlungspause bei parentalen Panc1 Zellen sowie nicht-CSCs und allen Panc89 Zellpopulationen eine Verringerung des zytostatischen Effekts zeigte. Des Weiteren spielte bei der sequentiellen Gabe von Zytostatika auch die Auswahl des Wirkstoffes, welcher als erstes verabreicht wurde, eine Rolle. Während alle Panc1 Zelllinien besser auf eine Vorbehandlung mit Oxaliplatin ansprachen, zeigte sich der größte Effekt auf alle Panc89 Zelllinien bei der Vorbehandlung mit Gemcitabin.

Zusammenfassend deuten die Ergebnisse dieser Arbeit auf die Existenz von unterschiedlichen CSC-/EMT-Phänotypen im PDAC hin, die zu der hohen Heterogenität innerhalb von Tumoren führen und aufgrund ihres divergenten Therapieansprechens unterschiedliche Behandlungen erfordern.

Summary

Pancreatic ductal adenocarcinoma (PDAC) is the most common malignant disease of the pancreas. Due to the late onset of non-specific symptoms, PDAC is usually diagnosed at an advanced or even metastasized stage, making it one of the most lethal tumor entities. The only curative option is surgical resection, which, however, is only considered for about 20% of PDAC patients. Nevertheless, even the majority of patients with resected tumors develop local recurrences or metastases within a few months to a few years. The rapidly developing resistance to current chemotherapeutic agents also complicates PDAC treatment. Therefore, research on the cause of the aggressive metastatic behavior and poor treatment response of PDAC is of great importance.

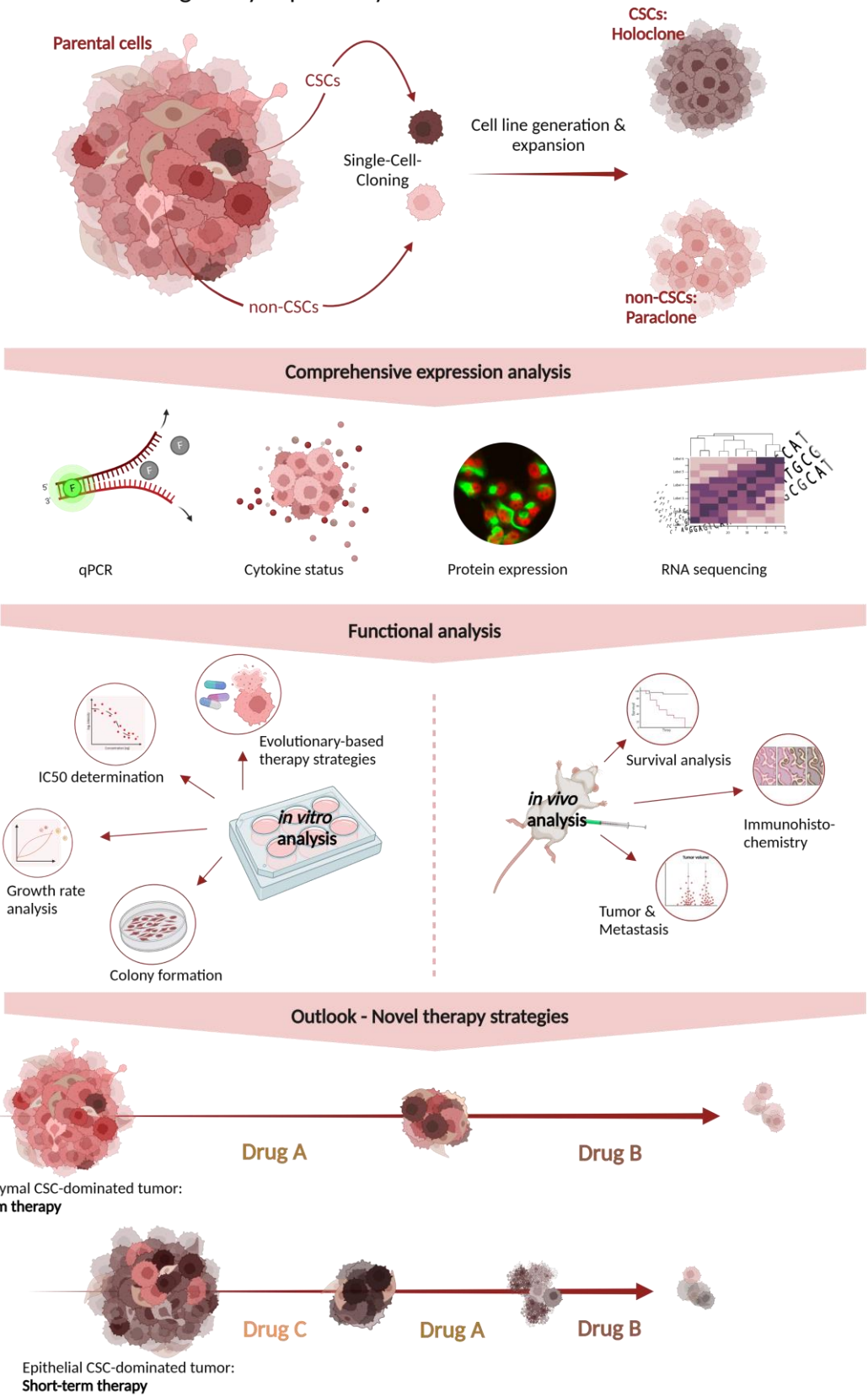
Cancer stem cells (CSCs) have the unique ability of self-renewal, unlimited proliferation and are able to give rise to different cancer cell phenotypes, including differentiated cancer cells. Furthermore, CSCs are characterized by their resistance to chemotherapies. This versatility of CSCs is related, among other things, to the process of Epithelial-to-Mesenchymal-Transition (EMT) and the reverse process of Mesenchymal-to-Epithelial-Transition (MET), in which epithelial cells with strong proliferative potential alternate their phenotype and gain mesenchymal characteristics with high invasion potential and *vice versa*. To date, it is not known whether CSC characteristics are always coupled to EMT/MET or whether there are different CSC phenotypes that also differ functionally. Therefore, the aim of the present study was to characterize different PDAC cell lines with respect to their CSC and EMT phenotype and to analyze these phenotypes regarding their therapy response. CSC clones and non-CSC clones were isolated from PDAC cells of different origins and analyzed comparatively with their parental cell line. For this purpose, Panc1 cells derived from a primary PDAC and Panc89 cells from a lymph node metastasis were used. Characterization revealed that epithelial and mesenchymal properties are coupled to different CSC factors and that a high heterogeneity between parental Panc1 and Panc89 cells as well as generated CSCs and non-CSCs exists. Panc1 CSCs showed high expression of the mesenchymal markers L1CAM and Vimentin, the EMT-inducer ZEB2, and the CSC-marker Nestin. The isolated Panc1 non-CSC clones showed a mesenchymal phenotype by expression of L1CAM and Vimentin but no expression of Nestin. In contrast, Panc89 CSCs exhibited high expression of the epithelial marker E-cadherin and the CSC-marker SOX2. Moreover, the Panc89 non-CSC clone showed an expression pattern indicative of a partial/hybrid EMT phenotype, as co-expression of E-cadherin, L1CAM and Vimentin

as well as the EMT-associated CSC-marker OVOL2 was detected. Functional analyses revealed an increased growth rate of all Panc89 cell lines compared to all Panc1 cell lines, with a higher growth rate of parental cells compared to isolated cell clones, except for the Panc89 CSC clone, which showed the same growth rate as the parental Panc89 cells. Examination of the metastatic potential *in vivo* revealed that tumors caused by Panc89 cell variants were larger and primarily localized in the peritoneum, compared to tumors formed by Panc1 cell variants, which were smaller and primarily detected in the pancreas. Furthermore, it was found that the number of CSC tumors formed was higher than the number of corresponding tumors of the non-CSC populations. With regard to survival, it was found that the median survival was shorter in animals inoculated with Panc89 CSC and non-CSC populations compared to Panc1 CSC and non-CSC injected animals which survived longer. With respect to treatment response, evolutionary biology treatment strategies were performed with alternating drugs and in the presence or absence of drug holidays. It was found that a drug holiday between two agents resulted in an increased treatment response only in Panc1 CSCs, whereas drug holiday showed a reduction of the cytostatic effect in parental Panc1 cells as well as non-CSCs and all Panc89 cell populations. Furthermore, the choice of which drug was administered first also played a role in the sequential administration of cytostatic drugs. While all Panc1 cell lines responded better to sequential treatment which started with Oxaliplatin, the greatest effect in all Panc89 cell lines was seen when Gemcitabine was applied first.

In summary, this work supports the view of the existence of distinct CSC/EMT phenotypes in PDAC which contribute to the high heterogeneity within this tumor entity and require different treatments due to their divergent treatment response.

Graphical abstract

PDAC: Tumor heterogeneity & plasticity



List of Abbreviations

Abbreviation	Definition
$\cdot g$	Unit of the centrifugal acceleration as a multiple of the mean gravitational acceleration g
μl	Microliter
μM	Micromolar
2D	two dimensional
5-FU	5-Fluorouracil
bp	base pairs
BRCA2	<i>Breast cancer 2</i>
BSA	Bovine serum albumin
CA	Canada/Kanada
CDKN2A	<i>Cyclin-dependent kinase inhibitor 2A</i>
cDNA	Complementary desoxyribonucleic acid
CFA	Colony-Formation-Assay/ Colony-Formation-Ability
CH	Switzerland/Schweiz
CSC	Cancer stem cell
CTRL	Control
ddH ₂ O	Distilled deionized water
DE	Germany/Deutschland
DH	Drug holiday (24 h)
dH ₂ O	Distilled water
DRA	Dose-Response-Analysis
EGFR	Epithelial growth factor receptor
EMT	Epithelial-to-Mesenchymal-Transition
EtOH	Ethanol
FCS	Fetal calf serum
G	Gemcitabine
GAPDH	Glycerinaldehyde-3-phosphate-dehydrogenase
GO-BP	Gene Ontology Biological Processes
GOI	Gene of interest
GSEA	Gene Set Enrichment Analysis
GSVA	Gene Set Variation Analysis
h	Hour
Holo	Holoclone
IC ₅₀	Half maximal inhibitory concentration
IF	Intermediate filament
IFS	Immunofluorescent staining
IHC	Immunohistochemistry
IL-8	Interleukin-8 (CXCL8)

IPMN	Intraductal papillary mucinous neoplasia
Isotype control mouse	IgG ms
Isotype control rabbit	IgG rb
KRAS	<i>Kirsten rat sarcoma viral oncogene homologue</i>
L-Gln	L-Glutamine
M	million
MCN	Mucinous cystic neoplasia
MET	Mesenchymal-to-Epithelial-Transition
MetOH	Methanol
mg	Milligram
ml	Milliliter
mM	Millimolar
mm²	square millimeters
mm³	cubic millimeters
ms	mouse
NaCl	Sodium chloride
NGS	Next generation sequencing
nM	Nanomolar
ON	Over night
Ox	Oxaliplatin
PA	Panama
PanIN	Pancreatic intraepithelial neoplasia
Para	Paraclone
PCA	Principal-Component-Analysis
PDAC	Pancreatic ductal adenocarcinoma
PFA	Paraformaldehyde
PI	Propidium iodide
POD	post-operative day
qPCR	Quantitative real-time polymerase chain reaction
rb	rabbit
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
s	second
SMAD4	<i>Small mother against decapentaplegic-family 4</i>
ST	Subtype
TF	Transcription factor
TGFβ	Transforming growth factor beta 1 (TGF-β1)
TNFα	Tumor necrosis factor alpha
TP53	<i>Tumor suppressor gene p53</i>

UK	United Kingdom/Vereinigtes Königreich
US	United States America/Vereinigte Staaten Amerika
v/v	Volume per volume
VEGF	Vascular endothelial growth factor

Table of Contents

Zusammenfassung.....	III
Summary	V
Graphical abstract	VII
List of Abbreviations.....	VII
Table of Contents	XI
1 Introduction.....	1
1.1 Pancreatic ductal adenocarcinoma	1
1.1.1 Epidemiology	1
1.1.2 Etiology.....	1
1.1.3 Pathogenesis	2
1.2 The metastatic cascade	5
1.2.1 Epithelial-to-Mesenchymal-Transition (EMT) and Mesenchymal-to-Epithelial-Transition (MET) in PDAC progression and metastasis.....	6
1.2.2 Cancer stem cells (CSCs) and plasticity	10
1.3 Chemotherapy: Mechanisms of action and treatment of PDAC	13
1.3.1 Current therapy of PDAC and evolution of therapy resistance	13
1.3.2 Adaptive and sequential therapy strategies	14
1.3.3 Chemotherapy in PDAC.....	15
1.3.4 Gemcitabine	16
1.3.5 Oxaliplatin	16
1.3.6 5-Fluorouracil	17
1.4 Aim of the study	18
2 Materials.....	19
2.1 Chemicals and reagents	19
2.1.1 Cytostatic agents	19
2.2 Cell culture.....	20
2.2.1 Cell lines.....	20
2.2.2 Cell culture medium	20
2.3 Molecular biological material.....	20
2.3.1 Primer	20
2.4 Immunobiological material	21

2.4.1 Antibodies for immunofluorescent staining	21
2.4.2 Antibodies for immunohistochemical staining	22
2.5 Consumables	23
2.6 Kit systems.....	23
2.7 Laboratory devices	24
2.8 Software	25
3 Methods	27
3.1 Cell biological methods <i>in vitro</i>	27
3.1.1 Cultivation of human pancreatic ductal adenocarcinoma cells.....	27
3.1.2 Long-term storage of pancreatic ductal adenocarcinoma cell lines.....	27
3.1.3 Cell counting <i>via</i> Neubauer chamber	28
3.1.4 Single-Cell-Cloning and cell clone expansion	28
3.1.5 Colony-Formation-Assay	29
3.1.6 Growth rate analysis	29
3.1.7 Determination of the half maximal inhibitory concentration for the cytostatic drugs Gemcitabine, Oxaliplatin and 5-Fluorouracil	29
3.1.8 Sequential treatment of parental Panc1 and Panc89, Holo- and Paraclone cells ..	31
3.2 Immunobiological & biochemical methods	32
3.2.1 Isolation and quantification of supernatants and proteins of PDAC cells	32
3.2.1.1 Multiplex analysis of cell culture supernatants	32
3.2.1.2 Protein isolation and quantification.....	33
3.2.2 Immunofluorescent staining	34
3.3 Molecular biological methods.....	35
3.3.1 RNA isolation and quantification	35
3.3.2 Complementary DNA synthesis <i>via</i> reverse transcription	36
3.3.3 Quantitative real-time polymerase chain reaction.....	37
3.3.4 RNA sequencing and transcriptomic analysis	38
3.4 Tumorigenicity assay <i>via</i> animal models <i>in vivo</i>	39
3.4.1 PDAC cell preparation and tumorigenicity assay	39
3.4.2 Immunohistochemical staining of tumors and tissues of mice inoculated with Panc1 or Panc89 Holo- or Paraclone cells.....	39
3.5 Statistical analysis.....	41
4 Results	42

4.1 Characterization of parental Panc1 and Panc89, Holo- and Paraclone cells revealed different colony formation ability	42
4.2 Characterization of gene and protein expression patterns in Panc1 and Panc89 cell lines	45
4.2.1 Transcriptomic and qPCR analysis of Panc1 and Panc89 cell variants.....	45
4.2.1.1 Principal Component Analysis of parental Panc1 and Panc89, Holo- and Paraclone cells showed distinct transcriptional signatures	45
4.2.1.2 Gene Set Enrichment Analysis revealed similarities in the molecular profile of Panc1 and Panc89 Holoclone cells	46
4.2.1.3 Gene Set Variation Analysis of parental Panc1 and Panc89 cells and derived Holo- and Paraclones revealed different regulation patterns of EMT, stemness and invasion	49
4.2.1.4 Marker gene analysis of parental Panc1 and Panc89, Holo- and Paraclone cells showed distinct EMT- and CSC-marker profiles.....	51
4.2.1.5 qPCR analysis revealed Panc1 Holoclone cells to exhibit a mesenchymal stemness phenotype while an epithelial stemness phenotype characterizes Panc89 Holoclone cells	52
4.2.2 Immunofluorescent staining of EMT- and CSC-markers confirmed gene expression pattern in parental Panc1 and Panc89, Holo- and Paraclone cells	56
4.3 Functional analysis of Panc1 and Panc89 cell lines	58
4.3.1 Panc89 cells showed enhanced cell growth rates compared to Panc1 cells	58
4.3.2 Panc1 and Panc89 cell clones essentially differ with respect to survival, tumor growth and metastatic capacity <i>in vivo</i>	61
4.3.3 Differences in EMT and CSC characteristics of Panc1 and Panc89 Holo- and Paraclone cells verified by immunohistochemical staining of Paraffin-embedded tissue sections.....	67
4.4 Different sequential treatments with cytostatic drugs of parental Panc1 and Panc89, Holo- and Paraclone cells	76
4.4.1 Determination of the half maximal inhibitory concentration of Gemcitabine, Oxaliplatin and 5-Fluorouracil for parental Panc1 and Panc89, Holo- and Paraclone cells <i>via</i> Dose-Response-Analysis	77
4.4.2 Short-term sequential therapies (72 h) of parental Panc1 and Panc89, Holo- and Paraclone cells with the cytostatic drugs Gemcitabine, Oxaliplatin and 5-Fluorouracil .	80
4.4.2.1 Short-term sequential therapy of parental Panc1 and Panc89, Holo- and Paraclone cells with two cytostatic drugs without drug holiday	80
4.4.2.2 Short-term sequential therapy of parental Panc1 and Panc89, Holo- and Paraclone cells with two cytostatic drugs with drug holiday.....	82

4.4.2.3 Short-term sequential therapy of parental Panc1 and Panc89, Holo- and Paraclone cells with three cytostatic drugs without drug holiday.....	85
4.4.3 Long-term sequential therapies (120 h) of parental Panc1 and Panc89, Holo- and Paraclone cells with the cytostatic drugs Gemcitabine, Oxaliplatin and 5-Fluorouracil .	88
4.4.3.1 Long-term sequential therapy of parental Panc1 and Panc89, Holo and Paraclone cells with three cytostatic drugs and without drug holiday.....	88
4.4.3.2 Long-term sequential therapy of parental Panc1 and Panc89, Holo- and Paraclone cells with two cytostatic drugs and with drug holiday.....	90
5 Discussion	95
5.1 Basal characterization of parental Panc1 and Panc89, Holo- and Paraclone cells revealed distinct CSC-EMT phenotypes	95
5.2 Distinct CSC/EMT phenotypes of mesenchymal and epithelial PDAC cell variants differ in their response to mono- and sequential therapy	101
5.3 Conclusion and outlook.....	106
6 References.....	110
Appendix.....	i
Supplementary data	i
List of Figures.....	xi
List of supplementary figures.....	xviii
List of tables	xxi
Erklärung	xxii
Danksagung	xxiii
Publikationsverzeichnis	xxv
Kongresse, Fortbildungen	xxvi
Auszeichnungen	xxvi

1 Introduction

1.1 Pancreatic ductal adenocarcinoma

1.1.1 Epidemiology

Pancreatic ductal adenocarcinoma (PDAC) is the most common malignant pancreatic tumor ¹. It is characterized by a poor prognosis with a five-year survival rate of around 12% and a still ongoing increase in death rate ¹⁻³. PDAC represents the 14th most common cancer entity and in 2018, 2.5% of all new cancer cases were diagnosed with PDAC and with 4.5% it ranks the 7th place of all cancer associated deaths worldwide ^{1,3,4}. The high mortality rate is mainly related to late and unspecific symptoms as well as lack of specific and early detection tests, therefore, the majority of patients are diagnosed at an advanced or even metastasized stage leaving only palliative treatment ⁵⁻⁷. The only curative option is surgery which, however, is only eligible to about 20% of PDAC patients ^{5,6}.

Metastatic sites of PDAC are predominantly organs localized nearby lymph nodes, like the liver, but also lung and peritoneum ⁷. Previous studies showed that resected patients with solitary pulmonary recurrences at the first site exhibit significantly longer disease-free survival and better overall prognosis in contrast to patients with liver and peritoneal recurrences ⁸. However, even patients that have undergone curative R0 pancreatectomy (tumor-free surgical margin) only show a median survival of about 12.6 months ⁹.

In Germany in 2018, approximately 19,000 people developed PDAC and due to the unfavorable prognosis, almost the same number of people died. The absolute number of new cases and deaths in Germany for both sexes have raised continuously over the last years, also due to the demographic development. With a proportion of 8.7% (woman) and 7.4% (men), respectively, it is the 4th leading cause of cancer deaths in both sexes. The mean age of onset for woman is 76 years and 72 years for men ⁴. Altogether, PDAC is estimated to become the 2nd most common cause of cancer deaths by 2030 ¹.

1.1.2 Etiology

Sporadic disease onset occurs in 90% of PDAC cases and is mainly attributed to lifestyle risk factors ^{4,10,11}. Confirmed life style risk factors include smoking, both active and passive, and severe overweight (obesity). Diabetes mellitus type 2, chronic inflammation of the pancreas (chronic pancreatitis, CP) and high alcohol consumption also increase the risk. Furthermore,

infections with pathogens such as *Helicobacter pylori*, hepatitis B or human immunodeficiency virus (HIV) are associated with the development of PDAC³. Overall, only about 10% of PDAC cases can be referred to hereditary components, e. g. hereditary pancreatitis is associated with cumulative risk in lifetime of 40-70% for PDAC development^{7,10,11}. First-degree relatives of pancreatic cancer patients are overall often more affected than average, associated to germline mutations in genes such as *Breast Cancer 2 (BRCA2)*, Figure 1)^{4,10}. The majority of CP cases are sporadic and not hereditary. Studies identified factors which are associated with a high risk of pancreatic cancer in sporadic CP. A low risk of pancreatic cancer development in sporadic CP was determined for metabolic syndrome, *Helicobacter pylori* infections, obesity, alcohol and red meat consumption, while long-term diabetes and smoking showed a moderate risk. A high risk could be determined for hereditary pancreatitis, germline mutations and CP¹²⁻¹⁴.

1.1.3 Pathogenesis

The pancreas is the main regulator of protein and carbohydrate digestion as well as glucose homeostasis. The exocrine pancreas makes up 80% of the organ mass and includes a branching network of acinar and duct cells, while the endocrine pancreas regulates metabolism and glucose homeostasis by hormone secretion into the blood stream. Physiologically, the organ is subdivided into pancreas head, neck, body and tail¹⁵⁻¹⁷. PDAC typically emerges from ductal epithelium of exocrine tissues of the pancreas head and comes along with infiltration of surrounding tissues like the lymphatic system and metastasis in liver, peritoneum and lungs^{6,18-21}. In the last decades, histological and molecular biological studies revealed three types of precursor lesions in pancreatic cancer, defined as mucinous cystic neoplasia (MCN), intraductal papillary mucinous neoplasia (IPMN) and pancreatic intraepithelial neoplasia (PanIN), whereby PanINs represent the best characterized premalignant lesions^{17,18,22,23}. PanINs can be distinguished into low-grade and high-grade dysplasia by stepwise progression from normal pancreatic ductal epithelium (Figure 1)²⁴.

Two stepwise cancer progression models are described in the literature. The first model is based on observations showing that higher degrees of precursor dysplasia correlate with higher accumulation of genetic alterations, possibly resulting in malignancy over years, similar to the development of other epithelial cancers^{22,25}. A more recent model describes punctu-

ated evolutionary progression and is based on findings, showing that PDAC driver gene inactivation can possibly occur simultaneously in chromosomal rearrangements, implying rapid tumor development and dissemination ²⁶.

Previous studies found that some normal ductal epithelial cells and approximately all low-grade PanINs harbor mutations of the *Kirsten rat sarcoma viral oncogene homologue (KRAS)* gene, while high-grade PanINs further contain tumor suppressor gene alterations, like a more frequent loss of *cyclin-dependent kinase inhibitor 2A (CDKN2A)*, *tumor suppressor gene p53 (TP53)* and *small mother against decapentaplegic-family 4 (SMAD4)*-member or hereditary *Breast Cancer 2 (BRCA2)* gene mutations (Figure 1) ^{10,18,24}. Nevertheless, these are just a few well-known mutations representing the genomic alterations acquired during PDAC development.

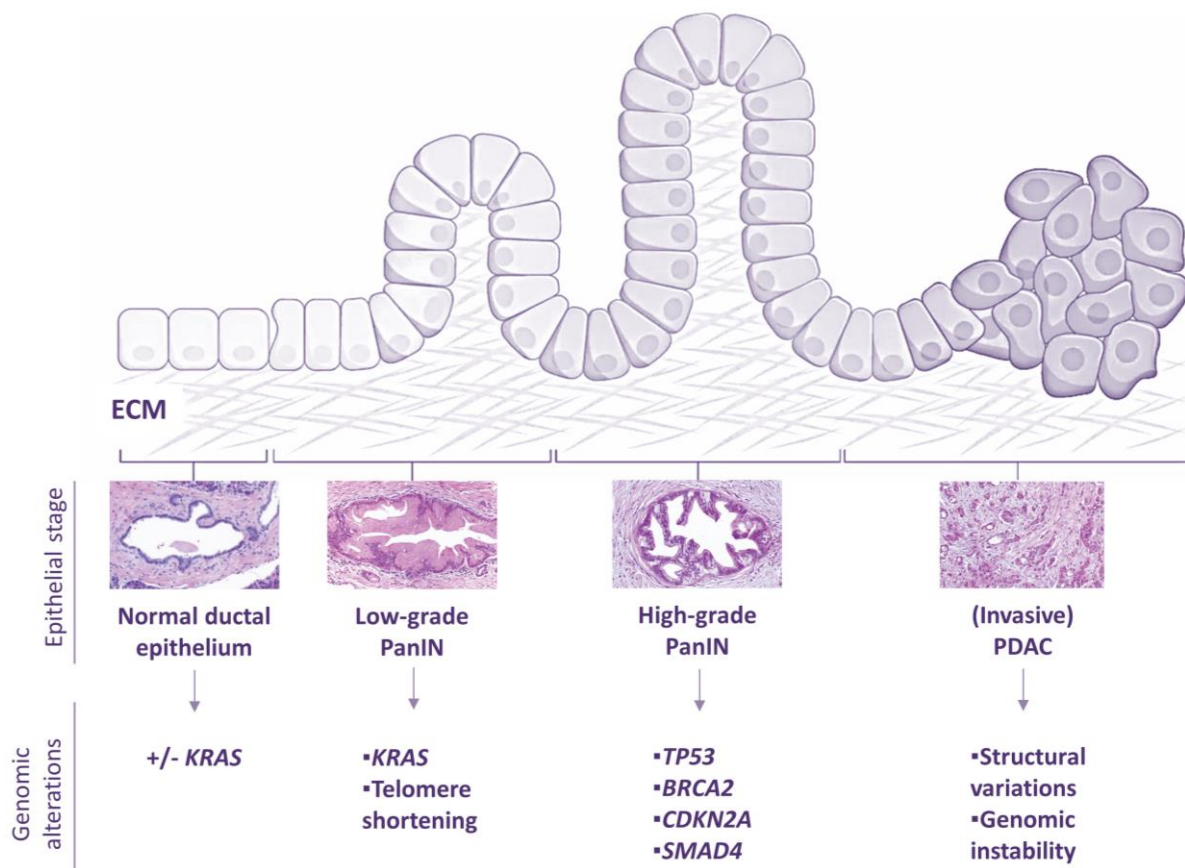


Figure 1 Progression of pancreatic ductal adenocarcinoma (PDAC) arising from pancreatic intraepithelial neoplasia (PanINs) is a multi-step process. The figure shows a schematic illustration of the progression from normal pancreatic ductal epithelium to low-grade and high-grade dysplastic PanINs and finally to (invasive) PDAC. Epithelial stages as well as genetic alterations during PDAC progression are shown. Low-grade PanINs bear early somatic changes in the *Kirsten rat sarcoma viral oncogene homologue (KRAS)* oncogene, while high grade PanINs can show inactivation of the *tumor suppressor gene p53 (TP53)* or underlying gene defects like *cyclin-dependent kinase inhibitor 2A (CDKN2A)*-, *small mother against decapentaplegic-family 4 (SMAD4)*-member or hereditary

Breast Cancer 2 (BRCA2) mutations. Invasive PDAC shows more structural variations and genomic instability. Adapted from ^{10,18,24}. (ECM = Extracellular matrix)

Subtyping of PDAC also plays an important role in pathogenesis and development of individualized therapy strategies. Different predominant transcriptomic- and surgical resection-based subtypes with different response behavior to chemotherapeutic regimens have been revealed by Collisson et al., Moffitt et al. and Bailey et al. ²⁷⁻²⁹.

Collisson et al. postulated a three-group classification into classical, quasi-mesenchymal and, exocrine-like PDAC subtypes ²⁷. The well-accepted two-group classification of Moffitt et al. validated the 1st tumor subtype as classical progenitor, and the 2nd subtype as basal-like ²¹. A four-group classification into squamous, immunogenic, pancreatic progenitor or aberrantly differentiated endocrine exocrine (ADEX) subtypes was identified by Bailey et al. ²⁹.

All three classification systems were directly compared in a study of The Cancer Genome Atlas (TCGA), where separate cohorts of 150 patients with resected primary PDACs were examined. The study revealed a transcriptional subtype classification for high-purity tumors into two groups, corresponding to the 1st with the quasi-mesenchymal group of Collisson et al., the squamous group of Bailey et al. or the basal-like group of Moffitt et al. and to the 2nd with the classical group of Collisson et al., the progenitor group of Bailey et al. or the classical group of Moffitt et al. ²⁷⁻³⁰.

Besides the transcriptional analysis it was found that PDAC can morphologically be classified by owing more than 40% gland formation and strong association with classical- or basal-like gene expression ³¹, while a squamous morphology, detected in over 30% of invasive tumors, was associated with basal-like characteristics in different studies ^{29,31,32}. However, it still remains to be elucidated which mechanisms lead to differentiation into these major subtypes during evolution of PDAC.

Moreover, the cancer cell surrounding tumor microenvironment, a hallmark of PDAC, plays a pivotal role in tumor progression, growth and metastatic properties as well as treatment resistance ³³. Sherman and Beatty 2023 described mesenchymal cells in the pancreas to be capable of controlling proliferative capacity of developing epithelial cells by secreting instructive signals that regulate morphogenesis. In the context of PDAC they showed that tumors comprise prominent mesenchymal compartments within the tumor stroma compared to other

solid tumor entities, which often account for most of the tumor volume. Tumor stroma includes *inter alia* extracellular matrix (ECM) components, activated fibroblasts and immune cells, strongly impacting the phenotype of neoplastic cells³⁴. Previous studies revealed that distinct microenvironment compositions influence tumor subregions that seemed to represent unique ecosystem-like niches within tumor lesions^{34,35}. These niches are termed based on their cellular infiltration status as reactive subregions (high cellular infiltration rate), or deserted subregions (low cellular infiltration rate). Whereas reactive subregions are associated with cellular stress response, deserted subregions are ECM signaling enriched. Furthermore, well-differentiated tumors could be found in deserted subregions, while moderately and poorly differentiated tumors were preliminary found in reactive subregions^{34,35}. These microenvironmental factors and ecosystem-like niches also impact the metastatic process as they influence cancer cell dissemination, seeding and colonization³⁶.

1.2 The metastatic cascade

One hallmark of cancer is the activation of invasion and metastasis³⁷. The complex multi-step process of metastasis starts with the acquisition of a motile and invasive phenotype followed by dissemination of tumor cells from the primary tumor into the surrounding tissue. Afterwards, tumor cells intravasate into blood and lymphatic vessels and by survival and circulation the tumor cells reach distant tissues. After extravasation of tumor cells from the lumina of different vessels into the parenchyma of secondary organs adaption to the novel environment starts. Colonization, proliferation and outgrowth of visible metastases is driven by reversion of the motile, mesenchymal phenotype, back to polarized, highly proliferating cells (Figure 2)³⁷⁻⁴⁰. It is suggested that cells of PDAC acquire pro-metastatic properties and already disseminate early during disease progression, possibly even before primary tumor formation or at least detection of the primary tumor starts⁴¹. This postulation is underlined by the fact that 30% of resected PDAC patients with negative microscopic tissue margins showed tumor relapse and distant metastasis within one year after surgery⁴¹⁻⁴³.

Furthermore, the ongoing process of metastasis and treatment failure is further aggravated by the fact that actively metastasizing PDAC cells with decreased cell division and slow cell proliferation may be less sensitive to treatment and cytostatic therapy, that typically targets fast proliferating cells. Thus, inhibiting metastatic invasion may in turn sensitize tumor cells to

different treatment strategies ^{44–46}. Overall, only tumor cells that are able to switch their phenotype (e.g., from a proliferating to a motile phenotype) will be able to leave the primary context and form metastases at secondary sites.

The metastatic cascade

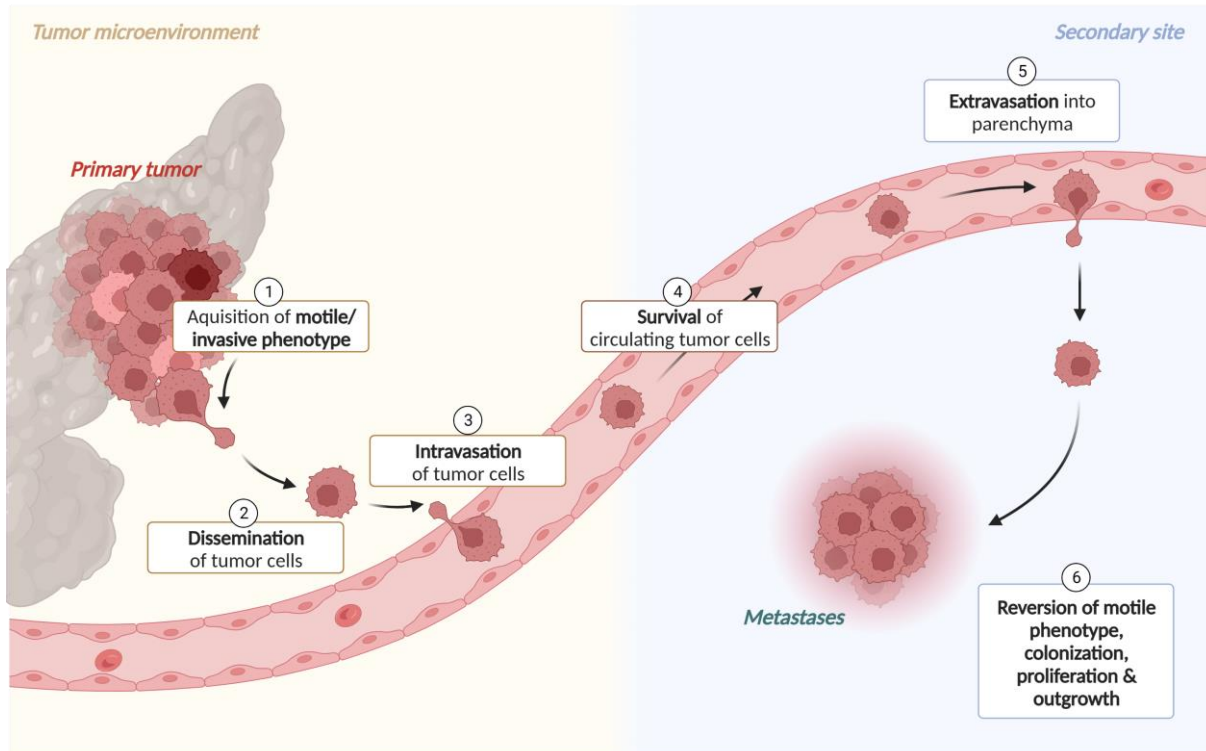


Figure 2 The metastatic cascade. This simplified illustration shows the multi-step process of metastasis, beginning with 1) the acquisition of a motile and invasive tumor cell phenotype followed by 2) the dissemination of tumor cells from the primary tumor, 3) intravasation of tumor cells into blood and lymphatic vessels, 4) survival of circulating tumor cells, 5) extravasation of tumor cells from the lumina of different vessels into the parenchyma of distant tissues and ending with 6) reversion of the motile phenotype, colonization of tumor cells, followed by proliferation and outgrowth of visible metastases at secondary sites. Created with BioRender.com.

1.2.1 Epithelial-to-Mesenchymal-Transition (EMT) and Mesenchymal-to-Epithelial-Transition (MET) in PDAC progression and metastasis

EMT and its reversion the MET are crucial physiological programs involved in early organ development and wound healing processes ^{47–49}. Besides, EMT is a regulatory process that plays a pivotal role in early steps of metastasis. EMT enables cells to disseminate, invade into surrounding tissues and form metastases at secondary sites ³⁷. The EMT program leads to the transition of highly proliferative, sessile epithelial cells to a more motile, mesenchymal-like

phenotype, coming along with the loss of apical-basal polarity, loss of epithelial cell-cell contacts, morphological changes, less proliferation, increased migratory properties and resistance to cell death (Figure 3) ^{37,50–53}.

MET plays a crucial role in later steps of metastasis and is associated with the ability of cancer cells to re-differentiate, adapt to new environments, regain proliferative properties and to form metastases at secondary sites (Figure 3) ^{47,54,55}. Of note, EMT and MET programs are not necessarily coupled. Roca et al. found that prostate cancer cells that formed metastases did not undergo the re-differentiating MET process and that these mesenchymal cells have the ability to proliferate and develop solid mesenchymal-like tumors ⁴⁹.

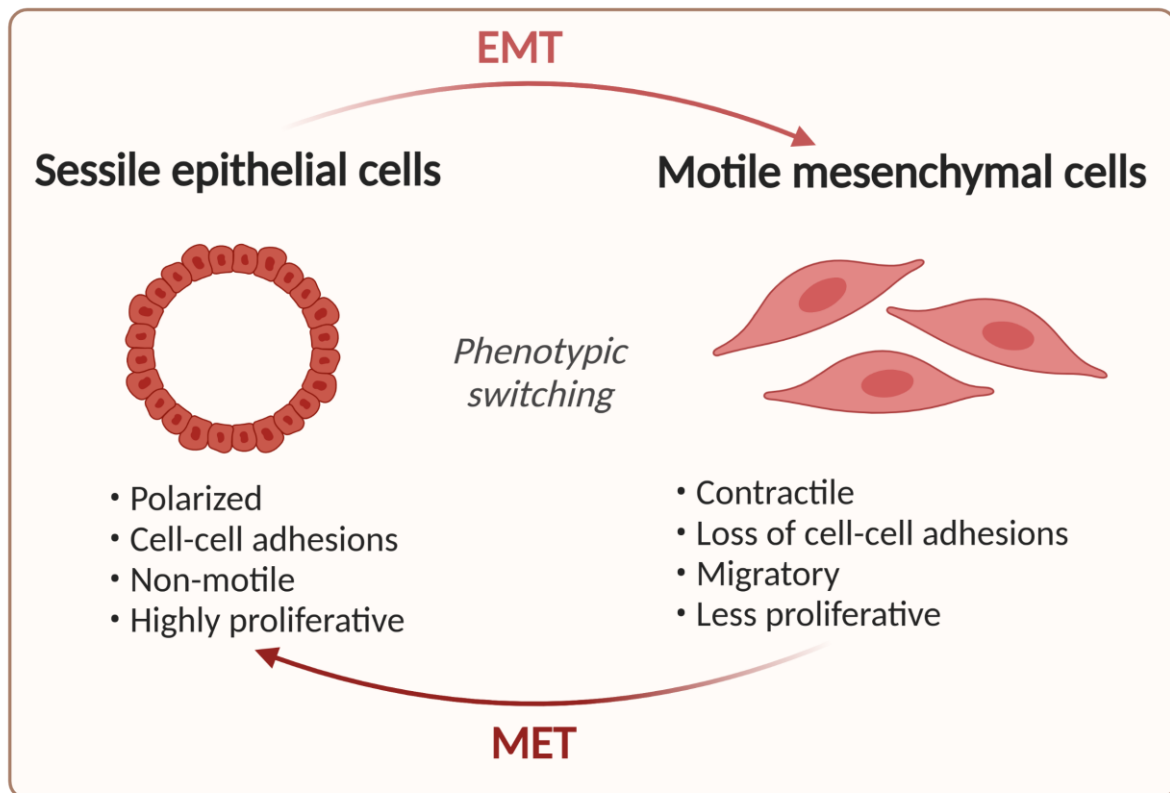


Figure 3 Simplified schematic illustration of cancer cell transition during Epithelial-to-Mesenchymal-Transition (EMT) and the reverse Mesenchymal-to-Epithelial-Transition (MET) process. The acquisition of an EMT phenotype enables cancer cells to disseminate, invade and form metastases at secondary sites. MET is associated with the reversion of invasive phenotypes, together with regaining proliferation capacity. In case of PDAC, ductal epithelial cells from primary sites lose their polarized, non-motile and highly proliferative phenotype and in turn, gain migratory and contractile characteristics, together with a loss of cell-cell adhesions and decreased proliferative potential. At secondary sites the motile, invasive characteristics are reversed to a highly proliferative, polarized phenotype leading the outgrowth of metastatic lesions. Created with BioRender.com.

In cancer progression, EMT is marked by transcriptional downregulation of epithelial proteins and adhesion molecules, like E-cadherin, and conversely a gain of mesenchymal proteins, like N-cadherin, Vimentin, L1CAM or the transcription factors (TFs) ZEB1, ZEB2, Snail and ZNF339^{49,51,52,56–59}. The EMT markers which are in focus of this work are introduced below.

E-cadherin, which is encoded by the *CDH1* gene, is a calcium-dependent, intercellular connecting membrane glycoprotein, playing an important role in cell adhesion, cell polarity and maintenance of cell-cell structures of epithelial cells. The major role of E-cadherin is its early expression at the two-cell stage of embryonic development and in adult tissue homeostasis^{53,60–62}. Downregulation or loss of E-cadherin expression is an initial step in trans-differentiation of epithelial cells and therefore, it is associated with tumor progression, invasion and metastasis⁶³. Moreover, in many adenocarcinomas, like PDAC, decreased E-cadherin expression correlates with an aggressive tumor types and poor survival^{60,64}.

The TFs **ZEB1** and **ZEB2** are encoded by *ZEB1* and *ZEB2* genes and are members of the Zinc finger-E-box (ZEB) family⁶⁵, with a key function in hematopoiesis and hematopoietic transformation^{66–68}. Physiologically, ZEB1 and ZEB2 control regulatory genes during embryonic development and cell differentiation^{67,69}. In tumor progression expression of ZEB1/2 represses E-cadherin transcription, induces EMT, impacts cell plasticity and promotes tumor invasiveness^{67,69–73}. Furthermore, ZEB1 and ZEB2 are required for maintenance of stem cell phenotypes and supporting a link between EMT and cancer stem cells (CSCs)^{74–77}. In line with these findings, a previous study found that depletion of ZEB1 promoted re-differentiation and sensitization to the cytostatic drug Gemcitabine in the resistant, mesenchymal PDAC cell line Panc1, revealing ZEB1 to be a crucial determinant of chemoresistance^{65,78}. Moreover, Galván et al. detected high levels of ZEB1 and ZEB2 in the tumor microenvironment of aggressive PDAC, especially in the surrounding of tumor-budding cells and invasive fronts. Interestingly, the study revealed two states of ZEB(1/2)^{+/–} tumor microenvironments. The 1st state was a ZEB1⁺ / ZEB2[–] phenotype of stromal cells associated with high EMT-phenotypic tumor-budding and invasiveness, while the 2nd state was the ZEB1[–] / ZEB2⁺ phenotype which was mainly found in cases with higher lymphatic invasion and presence of lymph node metastases⁷⁹.

Vimentin is a structural type III intermediate filament (IF) protein encoded by the *VIM* gene^{80,81} and it is one of the best studied members of the IF protein family. Vimentin is physiologically expressed in mesenchymal tissues and is known to regulate cellular integrity and

resistance against stress⁸². Furthermore, Vimentin plays a crucial role in regulating the metastatic cascade since Vimentin expression in various tumor entities correlates with increased metastatic potential and poor overall survival^{81,83–87}. Vimentin expression and its influence on EMT properties are induced by several factors, like Transforming Growth Factor beta1 (TGFβ1)⁸⁸ or ZEB2⁸⁹.

The neuronal cell adhesion molecule L1 (**L1CAM**) plays a pivotal role in a huge variety of diseases and has become a prominent research target over the last decades. L1CAM, a member of the immunoglobulin superfamily, is a cell surface protein mediating cell-cell interactions⁹⁰ and has been described as a cell surface antigen of the central nervous system⁹¹. Previous studies revealed a link between L1CAM and EMT properties by a disruption of E-cadherin-mediated adherens junctions and increased β-catenin-dependent transcription under increased L1CAM expression in human breast cancer cells⁹². Moreover, knockdown of L1CAM revealed a significant decrease in cell motility, examined by migration assays, while restoring of L1CAM expression completely recovered the former cell properties⁹². Overall, these data indicate the induction of L1CAM expression as part of the EMT process contributing to EMT properties in epithelial cells, by increased L1CAM expression during the EMT process followed by L1CAM mediated migration, tumorigenicity and resistance to apoptosis. Focusing on pancreatic cancer, expression of L1CAM elevates during disease progression in pancreatic ductal epithelium and is associated with poor prognosis of PDAC patients. Furthermore, L1CAM induces migration, resistance against apoptosis inducing stimuli and metastatic spread of premalignant pancreatic ductal epithelial cells and PDAC cells *in vitro* and *in vivo*^{93–96}.

However, this strict division of carcinoma cells into epithelial and mesenchymal phenotypes has been critically questioned during the last years. Recent studies on EMT suggested that this process is more a dynamic process with a broad spectrum of partial states between epithelial and mesenchymal cell types^{97,98}. This so called partial or hybrid EMT phenomenon suggests that cancer cells gain mesenchymal properties, while still maintaining other epithelial phenotypic traits. Partial/hybrid EMT has been captured in multiple malignancies like lung adenocarcinoma⁹⁹, breast cancer¹⁰⁰ and ovarian cancer¹⁰¹. Carstens et al. identified phenotypic clusters across the EMT spectrum in human and mice PDAC samples using single-cell RNA sequencing¹⁰². This work revealed tumor cell clusters that were characterized by either epithelial or mesenchymal cells, but also exhibit predominant partial/hybrid EMT characteristics¹⁰². Although there are already studies indicating that the partial/hybrid EMT stage is associated

with increased tumorigenicity, it remains to be elucidated whether a partial/hybrid EMT leads to stronger metastatic potential compared to a complete EMT phenotype in PDAC ^{48,53,99,103-108}.

1.2.2 Cancer stem cells (CSCs) and plasticity

Physiologically, stem cells (SC) have the ability to both self-renewal and cell differentiation, being capable to develop into a variety of different cell types ^{109,110}. Most tumors consist of a heterogeneous cancer cell population with different tumor initiating capabilities. Nevertheless, only a small number of distinct tumor cells were found to undergo symmetric and asymmetric cell division, thereby having self-renewal abilities and tumorigenic potential, which is why these unique cell populations are called CSCs ¹¹⁰⁻¹¹².

It is believed that asymmetric cell division of CSCs could give rise to intermediate CSCs like transit amplifying or hybrid CSC-like cells that are highly proliferative but lacking the stem-like properties ¹¹²⁻¹¹⁴. Owing to these unique features, CSCs are regarded as essential for tumor initiation at primary and secondary sites, including PDAC ¹¹⁵⁻¹¹⁷, to resist apoptosis induction by chemotherapeutic substances, and acquire a quiescent stage as common response ^{112,113,118-121}. Moreover, CSCs show exceptional organizing structures by recruiting and educating adjacent cells to provide nutrients and prevent immune system detection, leading to a favourable tumor growing environment ¹²². The EMT process and with this the gain of a de-differentiated phenotype has been described to be associated with the acquisition of CSC properties in carcinoma cells ^{109,123-125}. Thus, Mani et al. were the first who demonstrated that breast cancer cells that have undergone EMT acquired a stem cell-like phenotype, and likewise, stem cell-like cells resemble cells that have undergone EMT ¹²³. CSC populations within tumors can undergo symmetric and asymmetric cell division, show high self-renewal abilities and tumorigenic potential. Recent studies indicated that CSC properties can be gained and lost depending on the microenvironment ¹²⁵⁻¹²⁹, indicating that CSCs are not a stable, but highly plastic cell population. Thus, also non-stem like cells can acquire CSC characteristics, reflecting the strong plasticity potential of these cells.

Plasticity, defined as a phenotypical switch of cells by losing and gaining different cellular properties ¹³⁰, plays a pivotal role in a variety of physiological and pathological processes.

Physiologically, one of the best-known examples of plasticity is the transition of cells during gastrulation, where epithelial cells of the epiblast lose their apical-basal polarity and cell-cell-

junctions, and gain more mesenchymal-like properties to migrate through the primitive streak to build the embryonic germ layers ¹³⁰.

Pathologically, cellular plasticity can predispose tissues to cancerous transformation, impact cancer progression and therapy response ¹³⁰. Another crucial switch of the phenotype of cancer cells is metabolic plasticity, where cancer cells dynamically transit between different energy production pathways depending on internal or environmental factors. This metabolic plasticity impacts the adaption of cancer cells to changing microenvironments and thereby, also promotes migration and metastasis ¹³¹. In case of PDAC, the plastic switch between epithelial and mesenchymal phenotypes during the EMT/MET process is associated with metastasis ^{132–136}, epigenetic regulation ¹³⁷, therapy resistance ^{138–142} and CSC generation ^{143–146}. Interestingly, *ex vivo* models revealed that the tumor microenvironment of PDAC cells influences cell states and cell plasticity, independent of the cancer cell genotype, e.g. mutations in *KRAS* ¹⁴⁷. Plasticity does not only impact tumor cells but also stromal cells. Thus, cancer associated fibroblasts in PDAC are shown to be functionally and phenotypically different dependent on tumor microenvironmental subregions and niches they originate from, leading to a promoting or restraining tumor progression and proliferation ³⁴. In addition, CSCs are postulated to be highly plastic and therefore, play an important role within tumor formation and progression in a variety of cancers ¹⁰⁹. CSCs are able to maintain their silent, undetectable, resistant steady-state, while their transit amplifying progenitor cells continue to accumulate genetic changes during tumor proliferation and alter their epigenetic program *via* microenvironmental interactions. These mutational and epigenetic modifications confer to CSC characteristics. This in turn results in the generation of new CSC clones and promotes tumor cell heterogeneity within the whole tumor lifespan ^{112–114,148–150}. Moreover, CSCs are suggested to switch their proliferative and divisible cell state into dormant or quiescent stages, a process which is associated with treatment failure and therapeutic resistance ^{151–155}. Furthermore, a recent study revealed the ability of reprogramming chemotherapy-induced senescent cells into CSC-like cell states ¹⁵⁵. Senescent cells undergo growth arrest while maintaining metabolic activity, triggered by oxidative stress, oncogene activation, tumor suppressor loss, treatment with cytostatic drugs, tissue repair or aging ^{109,156–158}. Successful reversion of this cell state again reflects plasticity to be a crucial factor in tumor progression and therapy.

Overall, the current knowledge underlines heterogeneity and plasticity of PDAC cells to be of high relevance, but it remains poorly understood which factors impact plastic phenotypes and therefore, CSC properties, metastatic spread and therapy response.

Expression of several markers, e.g. ATP-binding cassette super-family G member 2 (ABCG2), CD133, CD24, CD44, Nanog, Nestin, ZNF339 and SOX2, has been proposed for CSCs in PDAC, indicating a strong heterogeneity of CSC populations ^{159–167}. The IF Nestin and the stem cell TFs SOX2 and ZNF339 seem to play a crucial role in the maintenance of CSC-properties ^{159,160,168,169} and were in the focus of this thesis.

The neuroepithelial stem cell protein **Nestin** is a class VI IF protein which is described as a neural stem cell marker and is encoded by the *NES* gene ¹⁷⁰. Nestin expression impacts cell motility and EMT-properties in PDAC cells and it has been shown that knockdown of *NES* in PDAC cells reduces tumor incidence and volume, as well as formation of metastases in a murine PDAC model ^{171,172}.

The TF **SOX2**, encoded by the *SOX2* gene, is the most investigated member of the sex determining factor Y (SRY)- family of proteins ^{173,174} and its expression is strongly related to maintenance of embryonic neural crest stem cell pluripotency and self-renewal ¹⁷⁵. Increased SOX2 expression in the pancreas has been associated with invasion and metastases of PanINs ¹⁷⁶. Furthermore, it has been shown that SOX2 is involved in the EMT process as knockdown of SOX2 in colorectal cancer (CRC) cells induced the MET process with recognized changes in expression of key genes involved in the EMT process including E-cadherin and Vimentin ¹⁷⁷. *De novo* SOX2 expression in PDAC cells is sufficient to promote self-renewal, de-differentiation and imparting stemness characteristics *via* impacting specific cell cycle regulatory genes and EMT-driver genes ¹⁶⁵.

Zinc finger protein 339 (ZNF339, referred to as **ZNF** from here on) is a TF which is encoded by the *OVOL2* gene. Zinc finger proteins are postulated to play a pivotal role in a variety of diseases like diabetes, CRC, breast cancer, prostate cancer, and PDAC ^{43,45,101,119,120}. Roca et al. demonstrated that ZNF expression is associated with MET mediation in a breast cancer model resulting in decreased cancer cell invasiveness. They found that cells expressing ZNF show significant changes in morphology, exemplified by a decreased number of cells with mesenchymal characteristics and increase of epithelial like cells ⁴⁹. Furthermore, ZNF has been demonstrated to inhibit migration and invasion of CRC and breast cancer cells through blocking EMT and that ZNF can regulate expression of ZEB1 *via* binding to its promotor ^{179–183}. Moreover,

ZNF was found to act as a phenotypic stability factor with the ability to maintain and stabilize cancer cells in hybrid/partial EMT states ^{103,104,107}.

The accounted plasticity of CSC and EMT characteristics in PDAC, which leads to a high heterogeneity within tumor cell populations, is also reflected by the ability to form different colony types *in vitro*, varying in their compositions of CSCs and differentiated cells. It is known that PDAC cells can generate three colony types with different morphologies ¹⁸⁴. While Holoclones, which are considered to be the colony type with the highest amount of CSCs, consist of small, densely packed, homogenous cells with a clear and regular boundary, Paraclones, which are characterized by more differentiated cells, are distinguished by scattered cells with irregular or divided boundaries ^{184,185}. Meroclones form an intermediate stage of these two colony types and differ from Holoclones mainly in their lower proportion of CSCs ^{184,185}.

1.3 Chemotherapy: Mechanisms of action and treatment of PDAC

1.3.1 Current therapy of PDAC and evolution of therapy resistance

In the case of resectable PDAC, surgery followed by adjuvant chemotherapy is the first choice of treatment. Adjuvant monotherapy with Gemcitabine or 5-Fluorouracil (5-FU) improves overall survival of patients for about six months compared to resection alone ^{186,187}. More recently, adjuvant therapies with combination regimens like FOLFIRINOX ^{188,189}, Gemcitabine together with nanoparticle-albumin (nab)-Paclitaxel ^{190–194} or neoadjuvant chemotherapy combined with or without radiotherapy before surgical resection has become more prominent ^{195,196}. Additionally, immunotherapy has opened new fields in cancer treatment, but failed to improve overall survival in PDAC yet ¹⁹⁷. Even though chemotherapy is fundamental and first-choice in PDAC treatment, in most cases therapeutic resistance occurs and treatment failure finally predominates ³⁴.

There are two types of cancer drug resistances: the intrinsic resistance (primary resistance) and the acquired resistance (also called secondary or adaptive resistance) ¹⁹⁸. The classical drug resistance hypothesis of Goldie Coldman, matching the secondary resistance type, states that mutations are spontaneously acquired by tumor cells over time, consequently leading to an accumulation of drug-resistant cell clones ¹⁹⁹. Cells with an EMT phenotype, CSCs, specific microRNAs, mutations in genes like *KRAS* or *BRCA1/BRCA2* as well as elevated expression of proteins like Nuclear Factor κ B (NF κ B), Epidermal Growth Factor Receptor (EGFR), Sonic

Hedgehog (SHH) and Vascular Endothelial Growth Factor (VEGF) are critical factors in mediating drug resistance^{200–206}. Additionally, elevated expression of ATP-binding cassette (ABC) transporters has been implicated in drug resistance, especially in pancreatic tumors, where ABC transporters are responsible for multidrug resistance against different types of cytostatic drugs like Gemcitabine and 5-FU^{207–212}. Furthermore, the tumor cell microenvironment (e.g., tumor-associated macrophages or cancer-associated fibroblasts^{213–215}) plays a pivotal role in mediating drug resistance by interaction of cell surface integrins, ECM components, metabolism of drugs and inducing NFκB expression/activity in PDAC cells^{216–218}.

EMT and CSCs are highly related, not only in PDAC progression and metastasis, but also in mediation of drug resistances^{139–142,209,219}. While undergoing the EMT process, the gain of mesenchymal properties along with CSC characteristics is associated with decreased apoptosis signaling and proliferation, leading to strong therapeutic resistance of cancer cells. Hence, as the EMT process has been linked to drug resistance, Shibue & Weinberg postulated three strategies to target the EMT program to improve cancer therapy: Preventing induction of the EMT process, selective targeting of cells that show active compounds of EMT, and reversing the process of EMT by forcing mesenchymal-like cancer cells to revert their phenotype into an epithelial-like cell state *via* activation of the MET program¹⁴². Even though there is a lack of reliable druggable targets due to PDAC tumor heterogeneity and plasticity, identification of EMT states as regulators of CSC phenotypes across different cancer types, could provide an opportunity to evaluate how CSCs and therapy resistance are coupled *via* EMT and how this could be targeted to improve cancer therapy^{142,220}.

In the future, combination therapies with different treatment regimens may become more important to improve overall survival and prognosis in PDAC treatment.

1.3.2 Adaptive and sequential therapy strategies

Drug discovery as well as development of novel treatment strategies play a crucial role in cancer therapy. Research in recent years focused on different evolutionary-based treatment strategies, like sequential and adaptive cancer therapies or the application of drug holidays. Previous studies reported that a longer drug holiday from chemotherapy in solid tumor treatments enhances the chance of drug response to re-treatments^{198,221–224}.

Sequential therapy works by administering drugs separately in a certain sequence and course aiming at a curative reduction of tumor burden. Adaptive therapy focuses on keeping a balance of sensitive and resistant cell clones, thereby, controlling the tumor burden which, however, might not lead to the cure of the patient ^{225–231}. Sequential therapy with different anti-tumor agents in high or low doses can improve therapeutic efficacy and reduce side effects, e.g. in patients with irresectable or metastatic PDAC, where treatment with Gemcitabine/nab-Paclitaxel or Leucovorin/Fluorouracil followed by FOLFIRINOX application showed feasible and promising therapeutic effects ^{229,232–238}. In turn, sequential treatment strategies aim at destroying the entire population of sensitive tumor cells, which again can impose selection for resistant cancer cells, maximize their proliferation, outgrowth and enhance therapy resistance, an evolutionary phenomenon which is well known as “competitive release” ^{231,239,240}. Adaptive therapy aims a stable tumor burden by allowing distinct cell populations of treatment-sensitive cells to survive and thereby, suppress proliferation of resistant cell populations ^{225,227,231}. Underscoring this strategy, mathematical modelling by Gatenby and colleagues predicted enhanced survival by replacing the “treatment-for-cure strategy” by the adaptive “treatment-for-stability strategy” ²²⁶. Both sequential and adaptive treatment strategies are promising therapeutic approaches but need to be further investigated, especially in case of PDAC. Here, the pronounced tumor heterogeneity and cell plasticity have shown to impede finding a suitable “one-for-all” strategy. Combining the most promising features of both sequential and adaptive therapies, like low-dose, tumor controlling drug concentrations applied in sequence added by drug holidays, could be more effective.

1.3.3 Chemotherapy in PDAC

Chemotherapy of cancer diseases can be divided into two major subgroups of processes based on the effects induced in the target cell: Cytotoxic drugs by activation/induction of cell death (apoptosis) and cytostatic drugs by inhibition of cell proliferation (growth arrest induction) and metabolism ^{241–245}. Of note, most chemotherapeutic agents are cytostatic drugs by function, thus, cytostatic drugs often induce cytotoxic effects, but not *vice versa*. The agents used in this work, Gemcitabine, Oxaliplatin and 5-FU, are cytostatic agents exerting cytotoxic, apoptosis inducing effects. Cytotoxic acting drugs target the DNA or metabolic steps playing a piv-

otal role in cell division and thereby, lead to cell death and reduce tumor burden. The following classes of cytotoxic drugs can be differentiated: Alkylating agents, antimetabolites, natural- or plant-based products and miscellaneous drugs.

1.3.4 Gemcitabine

Gemcitabine (2',2'-difluorodeoxycytidine), a member of the antimetabolite class, is a potent and specific deoxycytidine analog. It mediates its antitumor effects by fostering apoptosis in malignant cells that undergo DNA synthesis by blocking cell progression through G1/S-phase^{241–245}. After Gemcitabine uptake by malignant cells, it is phosphorylated by deoxycytidine kinase and forms Gemcitabine-monophosphate. Afterwards, it is converted into its active forms Gemcitabine-diphosphate and Gemcitabine-triphosphate which incorporate into the DNA chain, thereby inhibiting DNA chain elongation and preventing chain repair, a process which is denoted as “masked DNA chain termination”. Inserting of Gemcitabine triphosphate into the DNA chain finally leads to chain termination, DNA fragmentation and apoptotic cell death of actively proliferating cells^{246–248}. Gemcitabine has been used as first-line drug in PDAC treatment^{191,246,248} for both adjuvant and palliative monotreatment as well as in combination with other anti-cancer agents like nab-Paclitaxel (microtubule-damaging agent²⁴¹) or Erlotinib (tyrosine kinase domain inhibitor of the EGFR²⁴⁹)^{190,191,250}.

1.3.5 Oxaliplatin

Platinum-based agents, like Oxaliplatin, are not alkylating drugs by definition, but they are classified within this group because of the mechanistic and resistance similarities^{241–245}. Oxaliplatin was the first platinum-based anticancer drug that entered clinical use and belongs to the same chemotherapeutic drug family as Cisplatin and Carboplatin^{251,252}. Unlike other platinum-based drugs, Oxaliplatin forms fewer DNA adducts causing less side effects during treatment²⁵². Oxaliplatin selectively inhibits synthesis of DNA by undergoing nonenzymatic conversion to its active forms. After activation, it binds to the guanine and cytosine moieties which leads to a cross-linking of DNA. Thus, active forms of Oxaliplatin inhibit DNA synthesis and transcription, which finally leads to apoptosis^{251,253–255}. Oxaliplatin is a compound of the first-

line therapeutic drug regimen FOLFIRINOX (leucovorin calcium [folinic acid], fluorouracil, irinotecan hydrochloride, oxaliplatin), which is applied in PDAC patients with good health conditions as neoadjuvant, adjuvant and palliative treatment ^{189,191,250,251,256}.

1.3.6 5-Fluorouracil

5-FU, a pyrimidine analog belonging to the antimetabolite drug class, is commonly used in cancer treatment and is also part of the FOLFIRINOX regimen ^{241,257,258}. 5-FU works by inhibiting essential biosynthetic processes and normal functions of macromolecules like RNA and/or DNA ^{258,259}. In detail, the cytotoxic anticancer effects of 5-FU are caused due to inhibition of the thymidylate synthase (TS) by competing with deoxyuridine and by incorporation of its active metabolites into RNA and DNA and therefore, disrupting synthesis ^{258–260}. 5-FU rapidly enters the target cell by using the facilitated uracil transport mechanism. Indeed, cytotoxicity in host or tumor cells only proceeds by following anabolism in actively proliferating cells ^{259,261}. 5-FU was invented decades ago and is applied in a variety of cancers. In PDAC, 5-FU alone or together with Gemcitabine is used in adjuvant or palliative treatment and, in the combination regimen FOLFIRINOX, 5-FU is used as first-line therapeutic for patients with overall good health condition ^{186,187,257,258,260,262–265}.

1.4 Aim of the study

Despite intensive research effort during past decades, PDAC still remains an incurable malignancy and is estimated to become the second leading cause of cancer-related deaths by year 2030¹. PDAC evolves without specific symptoms and due to lack of early-detection methods, most of the patients are already in advanced or even metastasized stages at time of diagnosis, with palliative treatment being the last option⁵⁻⁷. PDAC is characterized by poor therapy response and emergence of therapy resistance, leading to treatment failure in most cases³⁴. The acquisition of EMT and CSC properties is regarded to be essential for tumor initiation at primary and secondary sites. Besides, the gain of CSC/EMT characteristics also implies the emergence of heterogenous, highly plastic cancer cell populations with the high probability to resist cell death induction by chemotherapy¹¹⁵⁻¹²¹.

This study aimed at an extensive and comprehensive characterization of clonal heterogeneity and plasticity in PDAC cell lines with a focus on CSC and EMT properties, and how these are related to functional phenotypes and therapy responses. Comparing CSC and non-CSC populations from mesenchymal and epithelial PDAC cell lines should provide a better understanding of cell compositions within a tumor cell population, and how clonal interaction of diverse cell populations could impact survival, tumorigenicity, metastatic behavior, and therapy response. For this purpose, two PDAC cell lines with different origins were analyzed. Panc1 cells emerged from a primary PDAC and showed mesenchymal characteristics, while Panc89 cells originated from a lymph node metastasis of PDAC and presented an epithelial phenotype. CSC as well as non-CSC clones of both PDAC cell lines were isolated *via* single-cell-cloning (SCC) and comprehensively analyzed. The characterization included a transcriptomic analysis followed by determination of CSC- and EMT-marker expression *in vitro* and *in vivo* on RNA and protein level, as well as analysis of growth rates, colony-formation ability, tumorigenicity, and responses to different treatment strategies. Here, it was focused on chemotherapeutic agents used in the clinical treatment of PDAC patients applied as monotherapy or in different sequential therapies to investigate whether PDAC cell clones with different CSC/EMT phenotypes respond differentially and whether selection and survival of resistant cell clones can be prevented.

In summary, this study aimed at a better understanding of heterogeneity and plasticity in PDAC cells and how the knowledge about different CSC/EMT phenotypes can be implemented to improve therapy response and overall survival of PDAC patients.

2 Materials

2.1 Chemicals and reagents

Compound	Manufacturer
Bovine serum albumin (BSA), fraction V	Biomol, Hamburg, DE
Crystal violet	Merck Millipore, Darmstadt, DE
Ethanol (denatured)	Merck Millipore, Darmstadt, DE
Ethanol (EtOH), ultrapure, 99.5%	Chemsolute® <i>via</i> TH. Geyer GmbH & Co. KG, Renningen, DE
Fetal calf serum (FCS)	Pan-Biotech GmbH, Aidenbach, DE
Fluor Save Reagent	Electron Microscopy Sciences, Hatfield, PA
Hoechst 33258 (1 mg/ml)	Merck Millipore, Darmstadt, DE
Hoechst 33342 (10 mg/ml)	Thermo Scientific, Schwerte, DE
Lämmli Buffer 10x	SERVA Electrophoresis GmbH, Heidelberg, DE
L-Glutamine (L-Gln), 200 mM	Pan-Biotech GmbH, Aidenbach, DE
LightCycler480 SYBR Green I Master	Roche, Basel, CH
Methanol	Th. Geyer GmbH, Renningen, DE
Paraformaldehyde (PFA) 4.0%	Thermo Scientific, Schwerte, DE
Phosphate-buffered saline (PBS) Dulbecco	Pan-Biotech GmbH, Aidenbach, DE
Propidium Iodide (PI) (1 mg/ml)	Merck Millipore, Darmstadt, DE
Roswell Park Memorial Institute (RPMI) 1640 medium	Pan-Biotech GmbH, Aidenbach, DE
Sodium Pyruvate, 100 mM	Pan-Biotech GmbH, Aidenbach, DE
Target Retrieval Solution, Citrate pH 6.1 (10x), S1699	DAKO Agilent, Santa Clara, US
Target Retrieval Solution, Tris/EDTA pH 9.0 (10x), S2367	DAKO Agilent, Santa Clara, US
Triton X-100	Sigma-Aldrich, München, DE
Trypsin-EDTA 10× in PBS	Biowest, Riverside, US

2.1.1 Cytostatic agents

Cytostatic	Carrier solution	Manufacturer
5-Fluorouracil (5-FU)	NaCl 0.9%	Medac – UKSH Kiel Pharmacy
Gemcitabine (G)	NaCl 0.9%	Hexal – UKSH Kiel Pharmacy
Oxaliplatin (Ox)	Bolus – no carrier solution	Kabi – UKSH Kiel Pharmacy

2.2 Cell culture

2.2.1 Cell lines

Cell line	Information/Reference
Panc1 (PANC-1)	Poorly differentiated human PDAC cell line isolated from the primary tumor of a 56-year-old Caucasian male. Panc1 cells are characterized by driver mutations in <i>KRAS</i> (G12D), <i>p53</i> (R273H) and <i>p16</i> (homozygous deletion) but show wildtype <i>dpc4</i> status. Panc1 cells were purchased from ATCC. ^{266–268}
Panc1 Holoclone	Cell clone with Holoclone characteristics, isolated from Panc1 parental cell population <i>via</i> single-cell-cloning. Cell clone #9, #15.
Panc1 Paraclone	Cell clone with Paraclone characteristics, isolated from Panc1 parental cell population <i>via</i> single-cell-cloning. Cell clone #5.
Panc89 (T3M-4)	Moderately differentiated human PDAC cell line isolated from a lymph node metastasis of 64-year-old Caucasian male. Panc89 cells are characterized by a <i>p53</i> mutation (Y220C) and methylated <i>p16</i> 5' CpG island but show <i>KRAS</i> and <i>dpc4</i> wild type status. Panc89 cells were kindly provided by K. Keiichi (Kyoto Prefectural University of Medicine, Japan). ^{266,267,269}
Panc89 Holoclone	Cell clone with Holoclone characteristics, isolated from Panc89 parental cell population <i>via</i> single-cell-cloning. Cell clone #4)
Panc89 Paraclone (3)	Cell clone with Paraclone characteristics, isolated from Panc89 parental cell population <i>via</i> single-cell-cloning. Cell clone #3.

2.2.2 Cell culture medium

Formula
RPMI 1640 medium (Supplemented: 10% (v/v) FCS, 1% L-Gln, 1% sodium pyruvate)

2.3 Molecular biological material

2.3.1 Primer

Target	5`- 3` Sequence	Annealing [C°]
<i>CDH1</i> (E-Cadherin)**	fw - TGCTCTTGCTGTTTCTTCGG rv - TGCCCCATTCGTTCAAGTAG	55
<i>GAPDH</i>*	fw - TCCATGACAACCTTTGGTATCGTGG rv - GACGCCTGCTTCACCACCTTCT	58
<i>L1CAM</i>**	fw - GAACTGGATGTGGTGGAGAG rv - GAGGGTGGTAGAGGTCTGGT	58
<i>NES</i> (Nestin)*	fw - GAAACAGCCATAGAGGGCAAA rv - TGGTTTTCCAGAGTCTTCAGTGA	58
<i>OVOL2</i> (ZNF339)**	fw - GGGACAAGCTCTACGTCTGC	58

- Materials -

	rv - GTCTGTCCTCCCCTTCCTTC	
SOX2*	fw - TCCCATCACCCACAGCAAATGA rv - TTTCTTGTCGGCATCGCGGTTT	58
VIM (Vimentin)**	fw - TCCAAGTTTGCTGACCTCTC rv - TCAACGGCAAAGTTCTCTTC	58
ZEB1*	fw - TCCATGCTTAAGAGCGCTAGCT rv - ACCGTAGTTGAGTAGGTGTATGCCA	61
ZEB2**	fw - CACATCAGCAGCAAGAAATG rv - AAACCCGTGTGTAGCCATAA	58

* = Primers purchased from Eurofins Genomics GmbH (Ebersberg, DE). Stocks of forward (fw) and reverse (rv) diluted at 1 pmol/μl in nuclease-free ddH₂O

** = Primers purchased from RealTime Primers (via Biomol, Hamburg, DE). Provided as stocks of 50 μM primer mix (containing fw and rv primer) in nuclease-free ddH₂O supplemented with 10 mM Tris-HCL and 0.1 mM EDTA (pH 7.5)

2.4 Immunobiological material

2.4.1 Antibodies for immunofluorescent staining

Target/Conjugate [Clone]	Host [Isotype]	Stock (μg/ml) [Dilution]	Manufacturer
- Isotype controls -			
Isotype [SP137]	Rabbit [IgG]	1000 [conditional]	Abcam, Cambridge, UK
Isotype [11711]	Mouse [IgG1]	500 [conditional]	R&D Systems GmbH, Wiesbaden, DE
- Primary antibodies (monoclonal) -			
L1CAM [L1-11A; UJ127.11]	Mouse [IgG1]	1000 [1:100]	Gerd Moldenhauer, German Cancer Research Center, Heidelberg, DE
Nestin [10C2]	Mouse [IgG1, κ]	1000 [1:200]	Thermo Scientific, Schwerte, DE
SOX2 [D6D9]	Rabbit [IgG]	24 [1:200]	Cell Signaling Technology, Danvers, MA - US
- Primary antibodies (polyclonal) -			
ZEB2	Rabbit [IgG]	100 [1:50]	Novus Biologicals, Wiesbaden Nordenstadt, DE
- Secondary antibodies -			
AlexaFluor488 – anti mouse IgG	Goat [IgG]	1000 [1:500]	Invitrogen, Carlsbad, US

- Materials -

AlexaFluor647 – anti rabbit IgG	Goat [IgG]	1000 [1:500]	Invitrogen, Carlsbad, US
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2.4.2 Antibodies for immunohistochemical staining

Target/Conjugate [Clone]	Host [Isotype]	Dilution	Manufacturer
- Isotype controls -			
Isotype	Mouse [IgG1]	1:80 (PanCK) 1:228 (L1CAM) 1:1484 (E-cadherin) 1:100 (Nestin)	Invitrogen <i>via</i> Thermo Fisher Scientific, Darmstadt, DE
Isotype	Mouse [IgG2a k]	1:275 (Vimentin)	DAKO Agilent, Santa Clara, US
Isotype	Polyclonal rabbit [IgG]	1:2500 (ZEB1) 1:5000 (ZEB2) 1:2000 (SOX2)	Abcam, Cambridge, UK
- Primary antibodies -			
E-cadherin [NHC-38]	Mouse [IgG1, κ]	1:100	DAKO Agilent, Santa Clara, US
L1CAM [L1-11A]	Mouse [IgG1]	1:1000	Peter Altevogt, German Cancer Research Center, Heidelberg, DE
Nestin [10C2]	Mouse [IgG1]	1:200	Merck, Darmstadt, DE
Pan-Cytokeratin [AE1/AE3]	Mouse [IgG1, κ]	Final concentration 12,7 µg/ml	DAKO Agilent, Santa Clara, US
SOX2 (brown)	Polyclonal rabbit [IgG]	1:20	Atlas Antibodies, Bromma, SE
Vimentin [Vim 3B4]	Mouse [IgG2a k]	1:232	DAKO Agilent, Santa Clara, US
ZEB1	Rabbit polyclonal [IgG]	1:100	Atlas Antibodies, Bromma, SE
ZEB2	Rabbit polyclonal [IgG]	1:100	Atlas Antibodies, Bromma, SE
- Secondary antibodies -			

- Materials -

Biotin	Poly goat anti-mouse [IgG, IgA, IgM]	1:200	LS-Bio, Massachusetts, US
Biotin	Poly goat anti-rabbit [IgG]	1:200	LS-Bio, Massachusetts, US
Dako REAL™ Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse, K5005	Biotinylated goat anti-mouse/anti-rabbit immunoglobulins	conditional	DAKO Agilent, Santa Clara, US

2.5 Consumables

Consumable	Manufacturer
0.1 - 10 µl, 10 - 200 µl, 100 - 1000 µl pipette tips	Sarstedt, Nümbrecht, DE
1.5 ml, 2.0 ml Eppendorf tubes	Eppendorf, Hamburg, DE
12-/24-/96-well clear flat-bottom plates	Sarstedt, Nümbrecht, DE
12-/24-/96-well clear flat-bottom plates	Greiner Bio-One GmbH, Frickenhausen, DE
15 ml, 50 ml centrifuge tubes	Sarstedt, Nümbrecht, DE
18 mm coverslips	Menzel, Braunschweig, DE
5 ml, 10 ml, 50 ml serological pipettes	Greiner Bio-One GmbH, Frickenhausen, DE
75 cm² cell culture flasks	Greiner Bio-One GmbH, Frickenhausen, DE
75 cm² cell culture flasks	Sarstedt, Nümbrecht, DE
96-well clear flat-bottom plates, Nunclon	Thermo Scientific, Schwerte, DE
96-well PCR plates, white	Roche, Basel, CH
96-well V-bottom plates	Greiner Bio-One GmbH, Frickenhausen, DE
CryoPure tube, 1.0 ml, white	Sarstedt, Nümbrecht, DE
Micro-fine U-100 Insulin syringe	Becton Dickinson, Heidelberg, DE
Micro-Touch nitrile examination gloves	Ansell GmbH, München, DE
Parafilm M sealing film	Brand GmbH & Co. KG, Wertheim, DE
SuperFrost Plus object slide 76x26 mm	Menzel, Braunschweig, DE
Transfer pipettes 3.5 ml	Sarstedt, Nümbrecht, DE

2.6 Kit systems

Kit	Manufacturer
Dako REAL™ Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse, K5005	DAKO Agilent, Santa Clara, US
DC™ Protein Assay	Bio-Rad Laboratories, München, DE
Illumina stranded mRNA Kit	Illumina, San Diego, CA
LEGENDplex™ CD8/NK Panel	BioLegend, Fell, DE
LEGENDplex™ Mix & Match System	BioLegend, Fell, DE

LEGENDPlex™ Total TGFβ1 Panel	BioLegend, Fell, DE
MycoAlert® Mycoplasma Detection Kit	Lonza Group Ltd, Basel CH
pegGOLD DNase I Digest Kit	PeqLab, Erlangen, DE
peqGOLD Total RNA Kit	PeqLab, Erlangen, DE
Quant-it RNA assay Kit	Thermo Fisher, Waltham, US
RevertAid First Strand cDNA Synthesis Kit	Thermo Scientific, Schwerte, DE

2.7 Laboratory devices

Centrifuges	Manufacturer
Heraeus Biofuge pico	Thermo Scientific, Schwerte, DE
Heraeus Fresco 17	Thermo Scientific, Schwerte, DE
Heraeus Multifuge 3 S-R	Thermo Scientific, Schwerte, DE
Heraeus Multifuge X1	Thermo Scientific, Schwerte, DE
Heraeus Pico 17	Thermo Scientific, Schwerte, DE
Mega Star 1.6	VWR International GmbH, Darmstadt, DE
Rotina 420 R	Hettich, Tuttlingen, DE
Sprout mini-centrifuge	Heathrow Scientific, Vernon Hills, US

Incubators/ Shaker	Manufacturer
BBD 6220 CO₂ incubator	Thermo Scientific, Schwerte, DE
HERA Cell 240 incubator	Thermo Scientific, Schwerte, DE
PMS 1000 microplate shaker	Grant Bio, Essex, UK
QBA1 block heater	Grant, Cambridge, UK
WNB 7-45 water bath	Memmert, Schwabach, DE
WTC ED-53 incubator	Binder, Tuttlingen, DE

Measuring Devices/ Microscopes	Manufacturer
AE2000	Motic, Wetzlar, DE
Axioscan Z1	Carl Zeiss AG, Jena, DE
BD FACSverse Flow cytometer	Becton Dickinson, Heidelberg, DE
CELLAVISTA® Scientific	Synentec, Elmshorn, DE
Evos XL Core Cell Imaging System	AMG, Bothell, US
Illumina NovaSeq6000 Flowcell	Illumina, San Diego, CA
Infinite 200PRO microplate reader	Tecan, Crailsheim, DE
LightCycler 480 II	Roche, Basel, CH
Lionheart FX Automated Microscope	BioTek, Bad Friedrichshall, DE
NanoQuant Plate	Tecan, Crailsheim, DE

- Materials -

Neubauer counting chamber	Marienfeld Superior GmbH & Co. KG, Lauda-Königshofen, DE
NYONE® Scientific	Synentec, Elmshorn, DE
Vevo 770	FUJIFILM VisualSonics Inc., Toronto, CA

Other Devices	Manufacturer
ARPEGE 110, liquid nitrogen tank	Air Liquide GmbH, Düsseldorf, DE
CS-300V power supply	Cleaver Scientific, West Sussex, UK
Freezer (-20°C)	Liebherr, Ochsenhausen, DE
Fridge (4°C)	Liebherr, Ochsenhausen, DE
HERAfreeze basic freezer (-80°C)	Thermo Scientific, Schwerte, DE
Laboport vacuum pump	KNF Neuberger, Freiburg, DE
Masterflex L/S tubing pump	Cole Parmer Instruments, Novodirect GmbH, Kehl/Rhein, DE
Sunrise remote control	Tecan, Crailsheim, DE
VORTEX Genius 3 vortex shaker	IKA-Werke, Staufen, DE

Pipettes	Manufacturer
Finnpipette F1 0.2-2 µl	Thermo Scientific, Schwerte, DE
Finnpipette F1 1-10 µl	Thermo Scientific, Schwerte, DE
Finnpipette F1 2-20 µl	Thermo Scientific, Schwerte, DE
Finnpipette F1 20-200 µl	Thermo Scientific, Schwerte, DE
Finnpipette F1 100-1000 µl	Thermo Scientific, Schwerte, DE
Macro pipette controller	Brand, Wertheim, DE
Pipetboy-acu	Integra Biosciences, Fernwald, DE
Ripette	Ritter, Schwabmünchen, DE

Sterile Benches/ Laminar Flow	Manufacturer
HERA Safe	Thermo Scientific, Schwerte, DE
HERA Safe KS	Thermo Scientific, Schwerte, DE

2.8 Software

Software	Manufacturer
BD FACSDiva Software	Becton Dickinson, Heidelberg, DE
Gen5 Data Analysis Software	BioTek, Bad Friedrichshall, DE
GraphPad Prism 9 (Version 9.5.0)	GraphPad Software, Californien, US
i-control Microplate Reader Software	Tecan, Crailsheim, DE
LEGENDplex Data Analysis Software v8	VigeneTech Inc., Carlisle, US

- Materials -

LightCycler480 Software	Roche, Basel, CH
Mendeley Desktop, v1.19.8	Mendeley Ltd, New York, US
Microsoft Office 2019	Microsoft Corporation, Redmond, US
YT®-Software	SYNENTEC GmbH, Elmshorn, DE
ZEN 3.3/ 3.5 (blue edition)	Carl Zeiss AG, Jena, DE

3 Methods

3.1 Cell biological methods *in vitro*

3.1.1 Cultivation of human pancreatic ductal adenocarcinoma cells

Human PDAC cell lines, Panc1 and Panc89 cells as well as their isolated Holo- and Paraclone cell variants, were cultured in 75 cm² cell culture flasks in 10 ml of their respective cell culture medium. The cells were split twice a week in a ratio of 1:3 – 1:5 for Panc1 cell lines or 1:5 – 1:15 for Panc89 cell lines, when cell cultures reached a confluence of about 80%. The adherent growing PDAC cells were detached by incubation with 5 ml Trypsin/EDTA at 37°C, 5% CO₂ and 86% humidity for 10 min (Panc1 cell lines) or 15-20 min (Panc89 cell lines). This proteolytic reaction was stopped by adding 5 ml of pre-warmed cell culture medium containing fetal calf serum (FCS). Afterwards, the cell suspension was transferred into a 50 ml centrifugation tube and cells were sedimented by centrifugation at standard cell culture program (300 ·g, 5 min, room temperature (RT)). The supernatants were aspirated and cells were resuspended in 10 - 15 ml of their respective cell culture medium. After dilution of the respective cell ratio, the cells were transferred into new 75 cm² cell culture flasks. Cell culture conditions for all cell lines were 37°C, 5% CO₂ and 86% humidity. All cell lines were regularly tested for mycoplasma (2.6), which were negative throughout the whole work.

3.1.2 Long-term storage of pancreatic ductal adenocarcinoma cell lines

For long-term storage of PDAC cell lines, cells were stocked in liquid nitrogen. For this purpose, the cells were centrifuged at standard cell culture program (3.1.1), supernatant aspirated and cells were resuspended in FCS containing 10% (v/v) DMSO to avoid crystallization. Afterwards, the cell suspension was poured in cryo tubes, transferred to a cryo-chamber filled with Iso-propanol to ensure a steady freezing, and stored at -80°C for at least 24 h. Finally, the cryo-tubes were transferred to a liquid nitrogen tank for long-term storage.

To take frozen cells into culture, the cell suspension needs to be thawed by resuspending with 37°C pre-warmed cell culture medium. After thawing, the resuspended cells were diluted in 10 ml fresh cell culture medium and sedimented *via* centrifugation at standard cell culture program (3.1.1). After discarding the supernatant, the cells were resuspended in respective cell culture medium and seeded to 75 cm² cell culture flasks.

3.1.3 Cell counting *via* Neubauer chamber

Counting of cells was performed manually *via* Neubauer counting chamber. Counting of cells is mandatory to seed appropriate cell numbers for the experiments. After trypzination, centrifugation and resuspension of detached cells (3.1.1), 10 µl of the resuspended cell solution were transferred into a the Neubauer counting chamber. Afterwards, four group squares were counted (dark cells without a bright lenticular boundary were neglected as dead cells) and cell numbers were calculated by the following formula:

$$\frac{\text{counted cell number}}{\text{counted group squares}} \cdot 10^4 = \frac{\text{cell number}}{\text{ml}}$$

3.1.4 Single-Cell-Cloning and cell clone expansion

To generate Holoclone (CSCs) and Paraclone (non-CSCs) cell lines from parental Panc1 and Panc89 cells, a single-cell-cloning (SCC) was performed. For this purpose, parental Panc1 and Panc89 cells were detached following the standard cell culture protocol (3.1.1) and diluted to a cell solution of 100 cells/ 20 ml. Thus, 200 µl of the cell solution contained one single cell, which were then transferred into each well of a flat 96-well plate. After centrifugation at standard cell culture protocol (3.1.1), the plates were imaged with the NYONE® Scientific and the corresponding *Single-Cell-Cloning*-operator of the YT®-Software and with the brightfield channel of EVOS microscope (2.7). Wells that were proven to contain only one single cell were imaged twice a week to analyze the colonies formed regarding their CSC or non-CSC morphology. Cell clones that reached a colony size minimum of 50 cells and with a stable colony morphology were detached from the 96-well plate and transferred to the well of a 6-well plate. The morphology of the cell clones was checked on a regular basis. Only clones with clear CSC or non-CSC morphology and stable phenotype were further cultivated, expanded and characterized. The phenotypic stability was examined by RNA analysis *via* qPCR periodically.

3.1.5 Colony-Formation-Assay

The examination of colony formation ability was performed by a Colony-Formation-Assay (CFA). After detachment of the cells by standard cell culture protocol (3.1.1), a cell dilution of 400 cells/ 2.5 ml respective cell culture medium per well was prepared and seeded as duplicates or triplicates in 6-well plates. After cultivation for eight to eleven days, the colonies were fixed with 4% PFA for 10 min, stained with 0.1% crystal violet (2.1) for 1 h, washed in dH₂O and air-dried at RT. Only colonies containing more than 50 cells were counted and their morphology type regarding Holo-, Mero- and Paraclones was determined. Holoclones consisted of tightly, homogenously clustered cells with a regular borderline, while Paraclones consisted of dispersed, larger cells with an irregular boundary ²⁷⁰. Meroclones defined by an intermediate colony type were neglected in this study ²⁷⁰.

3.1.6 Growth rate analysis

Parental Panc1 and Panc89 cell lines, as well as their Holo- and Paraclone cell lines were analyzed regarding their growth behavior. For this purpose, 0.5×10^4 cells of each cell line were seeded in a flat, transparent 96-well plate in triplicates and cell growth was monitored for 168 h (7 d). The monitoring was performed by imaging the cell confluence increase over time with the YT[®] *Confluence*-operator of the NYONE[®] Scientific imager. To analyze the number of nuclei, every day three replicate wells were dyed with Hoechst33342 (1:5000) and measured with the YT[®] *Nuclei-Count* operator of the NYONE[®] Scientific imaging device. Finally, data were analyzed in GraphPad Prism *via* nonlinear regression (curve fit) to maintain the growth rate (κ) for logistic growth.

3.1.7 Determination of the half maximal inhibitory concentration for the cytostatic drugs Gemcitabine, Oxaliplatin and 5-Fluorouracil

For the examination of the half maximal inhibitory concentration (IC₅₀) of the cytostatic drugs Gemcitabine (G), Oxaliplatin (Ox) and 5-Fluorouracil (5-FU) on parental Panc1 and Panc89, Holo- and Paraclone cells, a Dose-Response-Analysis (DRA) was performed. For this purpose, the cells were detached following the standard cell culture protocol (3.1.1) and seeded in duplicates in a flat, transparent 96-well cell culture plate with a cell number of 0.5×10^4 /well.

After an adherence time of 24 h, the treatment was started. The cell culture medium was removed and 200 µl of drug solvent control (NaCl or untreated; CTRL) or treatment solution was added to the cells. Nine different concentrations of the cytostatic drugs (Table 1).

Table 1 Concentrations of the cytostatic drugs Gemcitabine, Oxaliplatin and 5-Fluorouracil used for IC50 determination *via* Dose-Response Analysis (independent experiments n = 3-4)

Gemcitabine [µM]	
Panc1 (Parental, Holoclone, Paraclone)	Panc89 (Parental, Holoclone, Paraclone)
38,00000000	0,6000000000
3,800000000	0,0600000000
0,380000000	0,0300000000
0,038000000	0,0152500000
0,003800000	0,0076250000
0,000380000	0,0038125000
0,000038000	0,0019062500
0,000003800	0,0001906250
0,000000380	0,0000190625

Oxaliplatin [µM]	5-Fluorouracil [µM]
Panc1 & Panc89 (Parental, Holoclone, Paraclone)	
62,9300	76,880000
31,4600	15,380000
6,29000	7,690000
1,26000	3,840000
0,25000	1,920000
0,13000	0,960000
0,02600	0,480000
0,01300	0,050000
0,00650	0,005000

The cell confluence was determined before starting the treatment for defining the T0 value. Afterwards, cell confluence was measured every 24 h and analysis was terminated after 72 h with an endpoint analysis. The endpoint analysis was executed by live-dead-staining using Hoechst33342 (1:5000) and PI (1:50), carried out for 30 min at 37°C. Measurement of cell confluence as well as the live-dead staining was performed by use of the NYONE® Scientific

imaging device and the related YT®-Software. For the analysis of cell confluence, the YT® *Confluence*-operator was used, while the analysis of live-dead staining was performed *via* YT® *Virtual Cytoplasm*-operator. For the determination of IC50 values for the different cytostatic drugs, the endpoint measurement determined by the count of cell nuclei *via* Hoechst33342 staining was used. Finally, the IC50 values were calculated by GraphPad Prism (2.8) Nonlin-fit analysis for [inhibitor] vs. response (Table 2).

Table 2 IC50 values of Gemcitabine, Oxaliplatin, 5-Fluorouracil determined for Panc1 and Pan89 parental, Holo- and Paraclone cells by Dose-Response-Analysis (independent experiments n = 3-4)

Panc1			Panc89		
Parental	Holoclone	Paraclone	Parental	Holoclone	Paraclone
Gemcitabine [IC50]			Gemcitabine [IC50]		
0.0038 µM			0.0038 µM		
Oxaliplatin [IC50]			Oxaliplatin [IC50]		
0.5 µM	0.25 µM	0.5 µM	0.8 µM		
5-Fluorouracil [IC50]			5-Fluorouracil [IC50]		
0.8 µM	0.08 µM	0.4 µM	0.2 µM	1.5 µM	0.1 µM

3.1.8 Sequential treatment of parental Panc1 and Panc89, Holo- and Paraclone cells

The response of parental Panc1 and Panc89, Holo- and Paraclone cells to different sequential treatments with the cytostatic drugs G, Ox and 5-FU with or without drug holiday was examined. All treatment experiments were performed for either 72 h (short-term) or 120 h (long-term). All PDAC cell variants were seeded, following the standard cell culture protocol (3.1.1), into flat, transparent 96-well plates in duplicates with a cell number of 0.5×10^4 cells/well for 72 h settings and 0.25×10^4 cells/ well for 120 h settings. After an adherence time of 24 h, the T0 cell confluence was determined by NYONE® Scientific imager and related YT® *Confluence*-operator. Afterwards, the cell culture medium was removed and 200 µl of CTRL (cell culture medium from here on) or drugs were given to the cells. All treatments were carried out using the previously determined IC50 concentrations of the cytostatic drugs (3.1.7). Each cytostatic drug was sequentially combined with each other or applied in monotherapy.

Cell confluence measurements were terminated after 72 h or 120 h with an endpoint analysis. The endpoint analysis executed by live-dead-staining using Hoechst33342 (1:5000) and PI

(1:50) was carried out for 30 min at 37°C. Measurements of cell confluence as well as live-dead staining were performed in use of the NYONE® Scientific imaging device and the related YT®-Software. For the analysis of cell confluence, the YT® *Confluence*-operator was used, live-dead cell analysis was performed *via* YT® *Virtual Cytoplasm*-operator. Data are represented by endpoint nuclei-count analysis *via* Hoechst33342 staining. Statistical analysis was performed by 2-way ANOVA and Tukey's multiple comparison test. For this analysis, the significance output was only shown for P-values ≤ 0.0001 (***).

3.2 Immunobiological & biochemical methods

3.2.1 Isolation and quantification of supernatants and proteins of PDAC cells

3.2.1.1 Multiplex analysis of cell culture supernatants

Supernatants from parental Panc1 and Panc89, Holo- and Paraclone cells were analyzed for the abundance of cytokines by multiplex analysis *via* LEGENDplex™ Multi-Analyte Flow Assay Kits. LEGENDplex™ assays are bead-based flow cytometric immunoassays using the basic principle of sandwich immunoassays.

For this work, human LEGENDplex™ Mix and Match Subpanel and CD8/NK Panel and were carried out to determine the secretion of IL-8, IL-6, IL-10 and Tumor necrosis factor- α (TNF α). Analysis of supernatants of the different cell lines was performed with adapted antibody concentrations according to the manufacturer's instructions, and only 50% of the indicated volumes of supernatants and corresponding kit components were used.

Cell culture supernatants were collected after 24 h of cultivation and centrifuged for 10 min at 15.000 ·g and 4°C to remove remaining cells and cellular debris. Following experimental steps were performed by Silje Beckinger and Tina Daunke.

All reagents used for the assay were pre-warmed at RT prior to assay procedure. Pre-mixed beads and antibodies were incubated for 10 min in an ultrasonic bath and wash buffer was diluted with ddH₂O. The lyophilized standard was reconstituted in 250 μ l assay buffer by briefly vortexing and incubation for 10 min at RT. Then, the solubilized standard stock (C7) was diluted in a serial dilution with assay buffer to 1:4 (C6), 1:16 (C5), 1:64 (C4), 1:256 (C3), 1:1024 (C2) and 1:4096 (C1) and pure assay buffer was used as blank sample (C0).

Assay buffer was added to each well of a 96-well V-bottom plate as specified by manufacturer and afterwards, standard dilutions (C7 to C0) were given to the respective standard wells.

Corresponding cell culture supernatants were added to the respective sample wells. Pre-mixed bead cocktail was pipetted to each well and after sealing the plate with a plate sealer, it was wrapped in aluminum foil and incubated for 2 h at 800 rpm and RT on a plate shaker. Next, the plate was centrifuged for 5 min at 250 ·g at RT. Hereafter, the supernatant was dumped by quickly inverting and flicking the plate followed by adding 200 µl 1× wash buffer to each well. Then, the beads were resuspended by incubating the plate for 1 min at 800 rpm on the plate shaker. Afterwards, beads were again sedimented by centrifugation for 5 min at 250 ·g at RT. Next, 12.5 µl assay buffer and 12.5 µl detection antibody cocktail were added to each well. The beads were resuspended, the plate was sealed and wrapped in aluminum foil and incubated for 1 h at 800 rpm at RT on the plate shaker. Afterwards, 12.5 µl assay buffer and 12.5 µl SA-PE-Mix were added to each well, mixed and the plate was sealed, wrapped in aluminum foil and incubated for 10 min at 800 rpm at RT on the plate shaker. Finally, the plate was centrifuged for 5 min at 250 ·g at RT, the supernatant was discarded, beads were resuspended in 200 µl 1× wash buffer and transferred to a 1.5 ml Eppendorf tube for analysis. Data acquisition was carried out with a FACSverse flow cytometer and FACSDiva software. Data analysis was performed with the LEGENDplex™ Data Analysis software v8 according to the provided manual. The factor concentrations determined by the LEGENDplex™ analysis were normalized to the protein concentration of cultured cell lines.

3.2.1.2 Protein isolation and quantification

For the analysis of cytokines in supernatants of parental Panc1 and Panc89 as well as Holo- and Paraclone cells, proteins of the cultured cells were isolated and quantified as a reference for the cytokine concentrations determined in the LEGENDplex™ analysis (3.2.1.1).

For this purpose, cell culture medium was collected for further analysis (3.2.1.1) and cells were washed with PBS. Next, 100 - 150 µl Lämmli buffer were added to the cells depending on cellular confluence. After this detachment and lysis step, the cells were transferred into an 1.5 ml Eppendorf tube. Afterwards, the cell suspension was treated with a sonicator to ensure protein solubilization by cellular decomposition. Whole cell lysates were stored at -20°C.

Protein concentration was determined by colorimetric DC™ Protein Assay Kit provided by Bio-Rad according to manufacturer's instructions. Firstly, a standard row containing defined concentrations of BSA solved in Lämmli buffer was performed. Therefore, the provided BSA standard (1.55 mg/ml) was diluted 1:2, 1:4 and 1:8. Pure Lämmli buffer (zero value) and prepared

reaction buffer (blank value) were used as controls. Afterwards, 5 µl protein samples and standard row samples were applied in technical duplicates into a flat, transparent 96-well plate. Next, 25 µl of 1:50 dilution of reagent S and reagent A were added to each well, followed by 200 µl of reagent B. After an incubation time of 10 min in darkness at RT, the light absorption was measured at $\lambda = 750$ nm by use of the Tecan Infinite 200PRO spectrophotometer. Protein concentration was calculated *via* resulting regression equation for standard sample values.

3.2.2 Immunofluorescent staining

For the analysis of different CSC- and EMT-markers on protein level in parental Panc1 and Panc89, Holo- and Paraclone cells, an immunofluorescent staining (IFS) was performed. Based on RNA analysis data, parental Panc1 as well as Holo- and Paraclone cells were stained with anti-NESTIN and anti-ZEB2 antibodies, parental Panc89 cells and derived Holo- and Paraclone cells were stained with anti-SOX2 and anti-L1CAM antibodies.

Glass coverslips were placed in each well of a 12-well plate. Afterwards, detached cells were seeded with a number of 0.5×10^4 cells/well on to glass coverslips. After 48 h, cell culture medium was removed and coverslips were washed with PBS for 5 min. Fixation of the cells was performed by incubation with 4% PFA for 15 min at RT. Next, coverslips were washed 3×5 min with PBS and incubated with ice cold methanol (MetOH) for 10 min at -20°C for permeabilization of the cells. Afterwards, coverslips were washed again with PBS for 3×5 min. To reduce background staining originating from nonspecific protein-protein interactions, a blocking step was performed. For this purpose, cells were incubated with 4% BSA in 0.3% TritonX-100/PBS for 1 h at RT. After the following washing step with 0.3% TritonX-100/PBS for 3×5 min, incubation with primary antibodies was performed.

Concomitant double IFS staining of anti-NESTIN and anti-ZEB2 in Panc1 cell lines:

Every incubation of antibodies and Hoechst33258 was carried out in 1% BSA in 0.3% TritonX-100/PBS and in a humidity chamber. Incubation of the primary antibodies was performed at 4°C overnight (ON). The anti-NESTIN antibody was diluted 1:200 (= 5 µg/ml), anti-ZEB2 antibody was diluted 1:50 (= 2 µg/ml), the isotype CTRL mouse (IgG ms) was diluted 1:100 (= 5 µg/ml) and the isotype CTRL rabbit (IgG rb) was diluted 1:500 (= 2 µg/ml). Incubation of the primary antibody was performed as a double staining with both anti-NESTIN and anti-ZEB2 or the respective isotype CTRL antibodies at the same time. After ON incubation

with primary antibodies, the cells were washed with 0.3% TritonX-100/PBS for 3×5 min. For secondary antibody and nuclei staining, AlexaFluor488 (ms) antibody, AlexaFluor647 (rb) antibody and Hoechst33258, each diluted 1:500 in 1% BSA in 0.3% TritonX-100/PBS, were used. Incubation was performed for 1 h at RT. After washing the cells 3×5 min with PBS and 1×5 min with dH₂O, coverslips were sealed with Fluor Safe Reagent and transparent nail polish.

Sequential IFS staining of anti-SOX2 and anti-L1CAM in Panc89 cell lines:

Incubation of anti-SOX2 antibody and related secondary antibody were carried out in 1% BSA in 0.3% TritonX-100/PBS and in a humidity chamber. Incubation of anti-L1CAM antibody and related secondary antibody, as well as Hoechst 33258 staining were carried out in 1% BSA in PBS. Anti-SOX2 antibody was diluted 1:200 (= 25 µg/ml), anti-L1CAM antibody was diluted 1:100 (= 10 µg/ml), isotype CTRL mouse (IgG ms) was diluted 1:50 (= 10 µg/ml) and isotype CTRL rabbit (IgG rb) was diluted 1:40 (= 25 µg/ml). Incubation of primary antibodies was performed in sequence, started with the staining of anti-SOX2 at 4°C and ON. After incubation with the anti-SOX2 antibody or respective isotype CTRL antibody, the cells were washed with 0.3% TritonX-100/PBS for 3×5 min and incubated with AlexaFluor647 (rb; 1:500) conjugated antibody in 1% BSA in 0.3% TritonX-100/PBS for 1 h at RT. After washing the coverslips with PBS for 3×5 min, incubation with anti-L1CAM antibody or respective isotype CTRL antibody was carried out for 1 h at RT. After washing with 0.3% TritonX-100/PBS for 3×5 min, cells were incubated with AlexaFluor 488 (ms; 1:500) conjugated antibody and Hoechst33258 (1:500) in 1% BSA in 0.3% TritonX-100/PBS. Incubation was performed for 1 h at RT. After washing the cells for 3×5 min with PBS and 1×5 min with dH₂O, coverslips were sealed with Fluor Safe Reagent and transparent nail polish.

Image acquisition and staining evaluation for all cell lines were performed with the Lionheart FX Automated Microscope and related software.

3.3 Molecular biological methods

3.3.1 RNA isolation and quantification

RNA isolation from PDAC cell lines was performed with the column-based peqGOLD Total RNA Kit purchased from peqLab according to manufacturer's instruction. PDAC cell variants were washed with PBS and lysed with 300 µl lysis buffer T and thereby, detached from the culture plate. Afterwards, lysates were transferred to 1.5 ml Eppendorf tubes and frozen at -80°C for storage or directly subjected to RNA isolation.

The lysed cells were transferred to the DNA removing column and centrifuged at 12.000 ·g for 1 min and RT. The RNA containing flow through was mixed with 300 µl 70% (v/v) ethanol (EtOH), transferred onto the PerfectBind column and centrifuged at 11.000 ·g for 1 min at RT. The RNA bound to the column and flow through was discarded.

Optional DNase digestion was performed for samples which were subsequently used for RNA sequencing (3.3.4). For DNase digestion, 73.5 µl DNase buffer and 1.5 µl RNase-free DNase were mixed and transferred to the RNA containing PerfectBind column of the corresponding sample. The digestion was performed for 15 min at RT. Afterwards, 400 µl wash buffer I were added, incubated for 5 min at RT and centrifuged at 11.000 ·g.

After DNase digestion, the column was washed with 600 µl wash buffer II, centrifuged at 11.000 ·g and the empty binding column was dry-centrifuged again at 11.000 ·g. Finally, 40-80 µl RNase-free water were given onto the PerfectBind column and after 2 min incubation, the RNA was eluted into an 1.5 ml Eppendorf tube by centrifugation at 8.000 ·g for additional 2 min. Nucleic acid concentrations were analyzed using the Infinite 200PRO spectrophotometer together with respective NanoQuant plate application (2.7; 2.8). Long-time storage of RNA samples was carried out at -80°C.

3.3.2 Complementary DNA synthesis *via* reverse transcription

For synthesis of complementary DNA (cDNA) from isolated RNA samples, the RevertAid First Strand cDNA Synthesis Kit by Fermentas was used according to manufacturer's instructions. 0.1 ng – 500 ng RNA were pipetted into an 1.5 ml Eppendorf tube, 1 µl oligo dT primer was added and the suspension was filled up to a volume of 12.5 µl with nuclease-free water. The suspension was incubated for 5 min at 65°C, cooled-down on ice and short-spin centrifuged. Afterwards, 7.5 µl of master mix (Table 3) per sample were added, gently mixed, spun-down and incubated for 1 h at 42°C in the block heater. Finally, the samples were incubated for 5 min at 70°C to stop the reaction and cDNA was stored at -20°C.

Table 3 Master mix composition for cDNA synthesis from RNA templates per sample

Reagent	Volume
Reaction buffer (5x)	4 µl
RiboLock RNase inhibitor (20 U/µl)	0.5 µl
dNTP Mix (10 mM)	2 µl
RevertAid M-MuLV RvT (200 U/µl)	1 µl

3.3.3 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qPCR) for analysis of relative quantification of EMT- and CSC-marker expression was performed using the LightCycler 480 II and corresponding programs listed in Table 4.

Table 4 LightCycler program for cDNA amplification in qPCR reaction

Step	Time	Temperature (°C)	Process
1	5 min	95	Initial Denaturation
2	10 s	95	Denaturation
3	20 s	2.3.1	Annealing
4	30 s	72	Elongation
5	Steps 2-4 (40-50 cycles)		
6	10 min	72	Final elongation
7	5 min	72 - 95	Melting curve
8	∞	4	Cooling

Synthesized cDNA (3.3.2) was diluted 1:4 with nuclease-free water and 2.5 µl of this dilution were given into each well of a white 96-well PCR plate in duplicates or triplicates. Afterwards, 7.5 µl of respective master mix (Table 5) were added to each well and the plate was centrifuged for 2 min at 300 ·g and 4°C.

LightCycler SYBR Green I Master comprises FastStart Taq DNA polymerase, SYBR Green I dye, dNTPs and appropriate ionic concentrations/ components for a hot start reaction.

Table 5 Master mix composition for qPCR from cDNA templates per sample

Reagent	Volume (µl) (Eurofins Primer)	Volume (µl) (RealTime Primer)
Light Cycler SYBR Green I Master	5	5
Forward Primer	1	-
Reverse Primer	1	-
Forward/Reverse Primer Mix	-	1
Nuclease-free water	0.5	1.5

Evaluation of qPCR results was performed using the $2^{-\Delta\Delta C_t}$ -method. Here, the cycle threshold (Ct) was defined and the mean Ct-value of technical duplicates or replicates was calculated. For internal quality control, technical replicates with a Ct-value differing more than $\pm$

0.5 from each other were excluded from further analysis and repeated. Specificity of amplification process was verified by melting curve analysis. Finally, gene of interest (GOI) Ct-values were normalized to the respective Ct-value of the used reference gene Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) according to the following equation:

$$\text{relative gene expression} = 2^{-\left(\frac{Ct(GOI)}{Ct(GAPDH)}\right)}$$

3.3.4 RNA sequencing and transcriptomic analysis

Total high-purity RNA of parental Panc1 and Panc89 as well as Holo- and Paraclone cells was isolated using the total RNA kit peqGOLD with an additional DNase digestion step according to manufacturer's specifications (2.6). Afterwards, three independent biological RNA replicates of each cell line were placed in a flat, transparent 96-well plate at a concentration of at least 150 ng/15 µl RNase-free water and send to the Institute of Clinical Molecular Biology for transcriptomic analysis (Kiel, DE) for RNA sequencing, performed under supervision of Dr. Sören Franzenburg. Input total RNA was quantified using the Quant-it RNA Assay Kit and RIN scores were determined using the Agilent Tape Station. All RIN scores were > 8.200 ng input and total RNA was processed using the Illumina stranded mRNA kit. Libraries were sequenced on the Illumina NovaSeq 6000 SP Flowcell using 100 bp paired-end reads. Transcriptomic analysis of mRNA sequencing data was performed by Prof. Dr. Hauke Busch (Medical Systems Biology Group, Inst. of Experimental Dermatology, University of Lübeck, DE). Raw sequencing data (fastq format) was mapped against the human transcriptome (Ensembl GRCh38.106) using kallisto (v0.46.1) ²⁷¹, and gene expression was summarized from the scaledTPM values of the transcripts using tximport ²⁷². Differential gene expression was performed using DESeq2 ²⁷³. Gene set enrichment analysis (GSEA) on the shrunken log2 fold changes from apegIm ²⁷⁴ was performed using GAGE (v2.48.0) ²⁷⁵ against Hallmark, Reactome and Gene Ontology Biological Processes (GOBP) gene sets extracted from the msigdb R package (v7.5.1).

3.4 Tumorigenicity assay *via* animal models *in vivo*

3.4.1 PDAC cell preparation and tumorigenicity assay

All animal studies were executed in compliance with European guidelines for care and use of laboratory animals and approved by local authorities (V242-44598/2018 (80-8/18)). The tumorigenicity assay for the Panc1 Holo- and Paraclone cells was already performed in a previous study¹⁸⁴. Tumorigenicity assay for Panc89 Holo- and Paraclone cells was performed accordingly¹⁸⁴. PDAC cell variants were detached following standard cell culture protocol (3.1.1), tested for mycoplasma and cell number was calculated (3.1.3). 1×10^4 cells of either Panc1 and Panc89 Holo- or Paraclones were diluted in 75 μ l PBS and ingested into syringes. Anesthesia and surgery on animals were performed by Anne-Sophie Mehdorn, Jan-Paul Gundlach and Charlotte Hauser (UKSH, Kiel, DE). Progression of tumor formation was monitored regularly by palpation and abdominal ultrasound examination using Vevo 770 and was performed by Dr. Olga Will (MOIN-CC, Kiel, DE). At different time points, depending on tumor burden and general health condition of the mice, the animals were sacrificed by cervical dislocation. Afterwards, tumors as well as pancreas, livers and lungs were fixed in 4.5% PBS-buffered formalin and embedded in Paraffin for immunohistological examination.

3.4.2 Immunohistochemical staining of tumors and tissues of mice inoculated with Panc1 or Panc89 Holo- or Paraclone cells

Sectioning and immunohistochemical staining (IHC) of Paraffin-embedded tissues were performed at the University-Clinic Eppendorf Hamburg, Center for Experimental Medicine, Institute of Anatomy and Experimental Morphology, under the direction of Prof. Udo Schumacher. IHC sections were digitalized and followed by protein expression analysis using ZEN3.3 software. Sections were stained with antibodies against the CSC-markers NESTIN and SOX2, EMT-markers E-cadherin, L1CAM and Vimentin and EMT-inducers ZEB1 and ZEB2. ZNF staining could not be established during this work. Furthermore, Pan-Cytokeratin (PanCK) staining was performed for epithelial tumor tissue identification. Heat-mediated antigen retrieval (AR) conditions for all antigens are listed in Table 6.

Table 6 Heat-mediated antigen retrieval conditions for IHC analysis of mouse tissues

Antigen	Buffer (pH)	Temperature, Time
anti-E-Cadherin	S1699 (pH 6.1)	Steaming 121°C, 10 min
anti-L1CAM	EDTA (pH 8.0)	1. microwave broiling 800 watts 2. microwave 560 watts, 3×5 min
anti-Nestin	Citrate buffer (pH 6.0)	Steaming 121°C, 10 min
anti-PanCK	S1699 (pH 6.1)	1. Boiling 2. Waterbath, 95°C, 20 min
anti-SOX2	S1699 (pH 6.1)	Steaming 125°C, 4 min
anti-Vimentin	S2367 (pH 9.0)	waterbath 85°C, 20 min
anti-ZEB1	Citrate buffer (pH 6.0)	Steaming 121°C, 10 min
anti-ZEB2	Citrate buffer (pH 6.0)	Steaming 121°C, 10 min

Incubation of primary antibodies anti-PanCK, anti-L1CAM, anti-Vimentin, anti-ZEB1, anti-ZEB2, anti-NESTIN and anti-SOX2 was performed 1 h at RT (30 min for anti-E-cadherin), followed by 30 min incubation at RT for all secondary antibodies (15 min for ZEB2).

To quantify IHC staining of tumors, a tumor score was defined to characterize tumors in terms of percentage of positively stained cells (Frequency) and staining intensity (Intensity). The Frequency score was set from 1-4, Intensity score was set from 1-3 (Table 7).

Table 7 Frequency Score (= % of positively stained cells) and Intensity score (intensity of staining) for IHC analysis of mouse tissues

Frequency score	Intensity score
1 – Negative/ low (0 – 10%)	1 – Negative/ low
2 – Intermediate (11 – 50%)	2 – Intermediate
3 – High (51 – 90%)	3 – Strong
4 – Strong (> 90%)	–

3.5 Statistical analysis

Statistical evaluation was performed by GraphPad Prism 9. Ink (Version 9.5.0; 2.8).

All data sets were tested for normal distribution using Shapiro-Wilk test.

Parametric data including multiple groups were tested by one-way analysis of variance (one-way ANOVA) for statistical significance. Non-parametrical datasets of multiple groups were analyzed with Kruskal-Wallis one-way ANOVA on ranks test. Statistically significant differences between the groups were assumed at P-values ≤ 0.033 according to Tukey's method (parametric data) and Dunn's method (non-parametric data), respectively.

Graphs of parametric data were presented as mean with standard deviation or mean with standard error of means (depending on technical and biological replicates), while graphs of non-parametric data were presented as median with (interquartile) range.

T-test was used to examine two samples of normally distributed data.

Columned data sets were analyzed using one-way-ANOVA plus Tukey's multiple comparisons test for parametric data and Kruskal-Wallis test plus Dunn's multiple comparisons test for non-parametric data.

Growth rate experiments were analyzed *via* nonlinear regression (Nonlin curve fit) to maintain the growth rate (κ) for logistic growth.

Survival analysis of *in vivo* experiments was performed by Kaplan-Meier-Survival analysis with Mantel-Cox log-rank test for curve comparison.

Statistical significance was defined with p-value style NEJM at a P-value of ≤ 0.033 . Significances were defined using asterisks: $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$.

For the treatment analysis the significance output was only shown for $P \leq 0.0001 = ***$.

4 Results

4.1 Characterization of parental Panc1 and Panc89, Holo- and Paraclone cells revealed different colony formation ability

It is known that PDAC cells can generate three colony types with different morphologies and self-renewal capacities ¹⁸⁴. Holoclone colonies consist of relatively small, densely packed, homogeneous cells with strong cell-cell contacts and a clear colony boundary (Figure 4). Holo-clone cells are characterized by longevity, high proliferation rates, the potential to self-renew, expression of stem cell markers and increased resistance to chemotherapeutic drugs ^{184,185}. Contrary, Paraclone colonies are characterized by differentiated cells and a scattered, irregular colony shape without a clear boundary (Figure 4) ^{184,185}. Meroclone cells form an intermediate of both Holo- and Paraclone colonies, and differ from Holoclones mostly in their lower amount of CSCs ^{184,185}. From the two parental PDAC cell lines (Panc1 and Panc89), comprising a heterogeneous cell population with different cell clones, Holo- and Paraclone cell lines were generated by SCC and expanded. First, the colony formation ability, being indicative for the cellular self-renewal properties of the different parental Panc1 and Panc89, Holo- and Paraclone cell variants, was comparatively analyzed. Focussing on Holo- and Paraclones of both PDAC cell lines, the intermediate colony types (Meroclones) were neglected. Typical colony morphologies of parental Panc1 and Panc89 as well as their Holo- and Paraclone cells are shown in Figure 4.

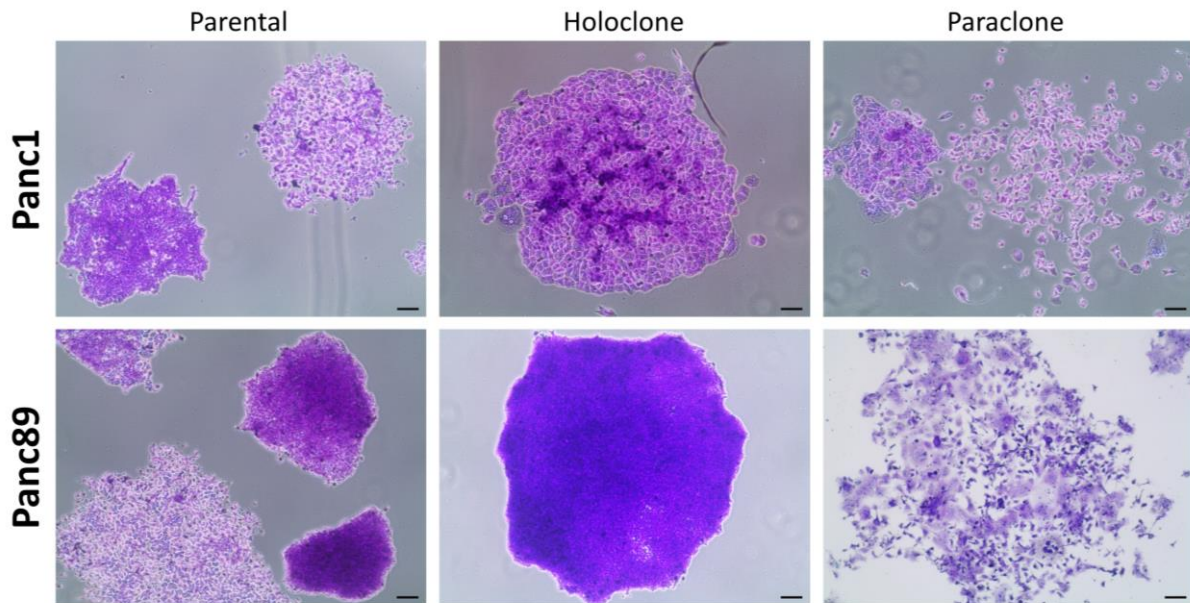


Figure 4 Representative Colony-Formation-Assay images of colonies formed by parental Panc1 and Panc89 cells and there of derived Holo- and Paraclone cells. Parental Panc1 and Panc89 as well as Holo- and Paraclone cells were analyzed regarding their colony-formation ability, being indicative for the cellular self-renewal properties, *via* Colony-Formation-Assay (CFA). Holoclones consisted of tightly, homogenously clustered cells with a regular boundary, while Paraclones consisted of dispersed, larger cells with an irregular boundary. Parental cells are characterized by a heterogeneous cell population comprising both Holo- and Paraclones. Scale bar = 100 μ m.

The total number of colonies formed per well by Panc1 Holo- and Paraclone cells was lower compared to Panc89 Holo- and Paraclone cells, while the total number of colonies formed per well by parental Panc1 and Panc89 cell variants was comparable (12 and 11, respectively). Parental Panc1 cells formed 2 Holo- and 10 Paraclone colonies, while parental Panc89 cells gave rise to 8 Holo- and 3 Paraclone colonies, indicating that in the parental Panc1 cell population Paraclones dominate, while in parental Panc89 cells rather Holoclones are the dominant cell clone. Panc1 Holoclone cells formed a total of 9 colonies and Panc1 Paraclones formed 11 colonies per well. Contrary, Panc89 Holoclone cells formed a total of 102 colonies, presenting a ~10-fold increase compared to Panc1 Holoclone cells, and Panc89 Paraclones formed 51 colonies per well, still presenting a ~5-fold increase compared to Panc1 Paraclone cells (Figure 5). Regarding the colony type it could be determined that parental Panc1 and Paraclone cells predominately formed Paraclone colonies, while Panc1 Holoclone cells, even though few colonies were formed overall, primarily gave rise to Holoclone colonies (Figure 5 A). On the other hand, parental Panc89 cells as well as Holo- and Paraclones, primarily formed Holoclone colonies (Figure 5 B). However, Panc89 Holoclone cells brought up the highest number of colonies ($n = 102$), from which most of the colonies were Holoclones ($n = 96$) and only 6 Paraclone

colonies (Figure 5 B). Compared to these results, Panc1 Holoclone cells formed a total amount of 9 colonies, consisting of about 8 Holoclone colonies and 1 Paraclone colony. Overall, these data indicate a stable CSC phenotype of both Panc1 and Panc89 Holoclone cells (Figure 5 A). Furthermore, Panc1 Paraclone cells formed a total of 11 colonies, all of them exhibiting a Paraclone morphology, while Panc89 Paraclone cells gave rise to 35 Holo- and 16 Paraclone colonies. In contrast to Panc1 Paraclone cells, which showed a stable phenotype, no uniform Paraclone phenotype could be detected in Panc89 Paraclone cells, which may indicate a high plasticity of this cell population.

Overall, these findings indicate a high heterogeneity between both parental PDAC cell lines, but also between the generated Holo- and Paraclone cells regarding the self-renewal capacity.

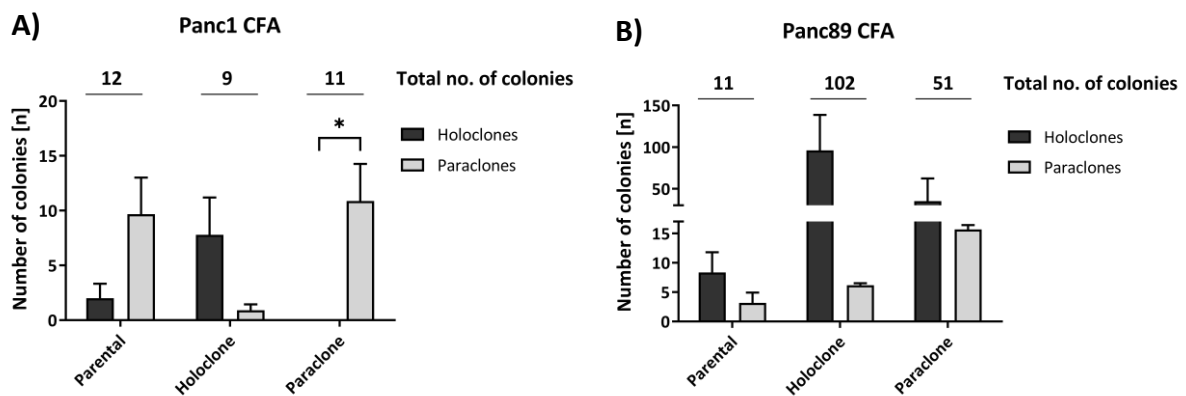


Figure 5 Parental Panc1 and Panc89 as well as their Holo- and Paraclone cells show different colony formation ability. Parental Panc1 and Panc89 cells and their Holo- and Paraclone cells were analyzed regarding their colony-formation ability *via* Colony-Formation-Assay (CFA). 400 cells of each cell line were seeded in duplicates/triplicates in 6-well plates and after eight to eleven days, the colonies were fixed with PFA and stained with crystal violet. The colonies formed were morphologically characterized regarding Holo- and Paraclones. Number of formed Holo- and Paraclones per well are shown for A) parental Panc1, Holo- and Paraclone cells as well as B) parental Panc89, Holo- and Paraclone cells. Y-axis in B) is segmented with 0-18 for the bottom part and 30-200 for the upper part. Data were analyzed with two-way ANOVA and are shown with standard error of means. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$ (independent experiments $n = 3-7$).

4.2 Characterization of gene and protein expression patterns in Panc1 and Panc89 cell lines

4.2.1 Transcriptomic and qPCR analysis of Panc1 and Panc89 cell variants

Next, a transcriptomic characterization of parental Panc1 and Panc89 cell lines and isolated Holo- and Paraclone cells was performed using mRNA sequencing and quantitative real-time polymerase chain reaction (qPCR).

4.2.1.1 Principal Component Analysis of parental Panc1 and Panc89, Holo- and Paraclone cells showed distinct transcriptional signatures

For transcriptomic analysis, a Principal Component Analysis (PCA) was carried out first. PCA projects high-dimensional data onto a low-dimensional space spanned by principal components. The projection preserves the maximum amount of information while enabling its visualization. Here, the high-dimensionality arises from measuring the expression strength of about 20,000 transcripts per sample. The projection onto two principal components then allows to estimate the transcriptome similarity and trends in the distinguishing features ²⁷⁶. Figure 6 depicts the PCA for parental Panc1 and Panc89, Holo- and Paraclone cells for the first two principal components, which arranges the samples along the highest (PC 1) and orthogonally second highest variation (PC 2), respectively. The PCA of the parental Panc1 and Panc89 as well as Holo- and Paraclone cell samples separated all cell variants along PC1 with a variance of about 77%. Holo- and Paraclone populations from both Panc1 and Panc89 separated with a much lower variability of about 7% along PC2. These findings indicate distinct transcriptional signatures for parental Panc1 and Panc89 cells and consistent but smaller gene expression changes for the Holo- and Paraclones, irrespective of their origin from Panc1 or Panc89 cells.

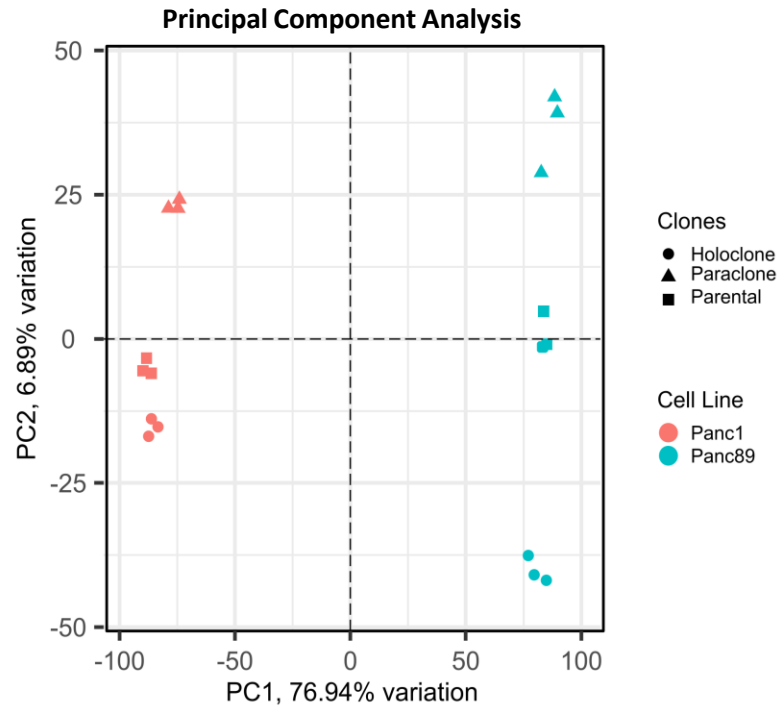


Figure 6 Principal Component Analysis based on the 10% most varying transcripts across all of parental Panc1 and Panc89, Holo- and Paraclone cell samples using the R package PCAtools (v2.10.0). Principal Component Analysis (PCA) was performed for the highest variation in the first principal component (PC1) on the X-axis, and for the lower variations by the second principal component (PC2) on the Y-axis. All cell variants are denoted by color (red = Panc1 cell variants, blue = Panc89 cell variants) and symbol, respectively. Independent replicates $n = 3$.

4.2.1.2 Gene Set Enrichment Analysis revealed similarities in the molecular profile of Panc1 and Panc89 Holoclone cells

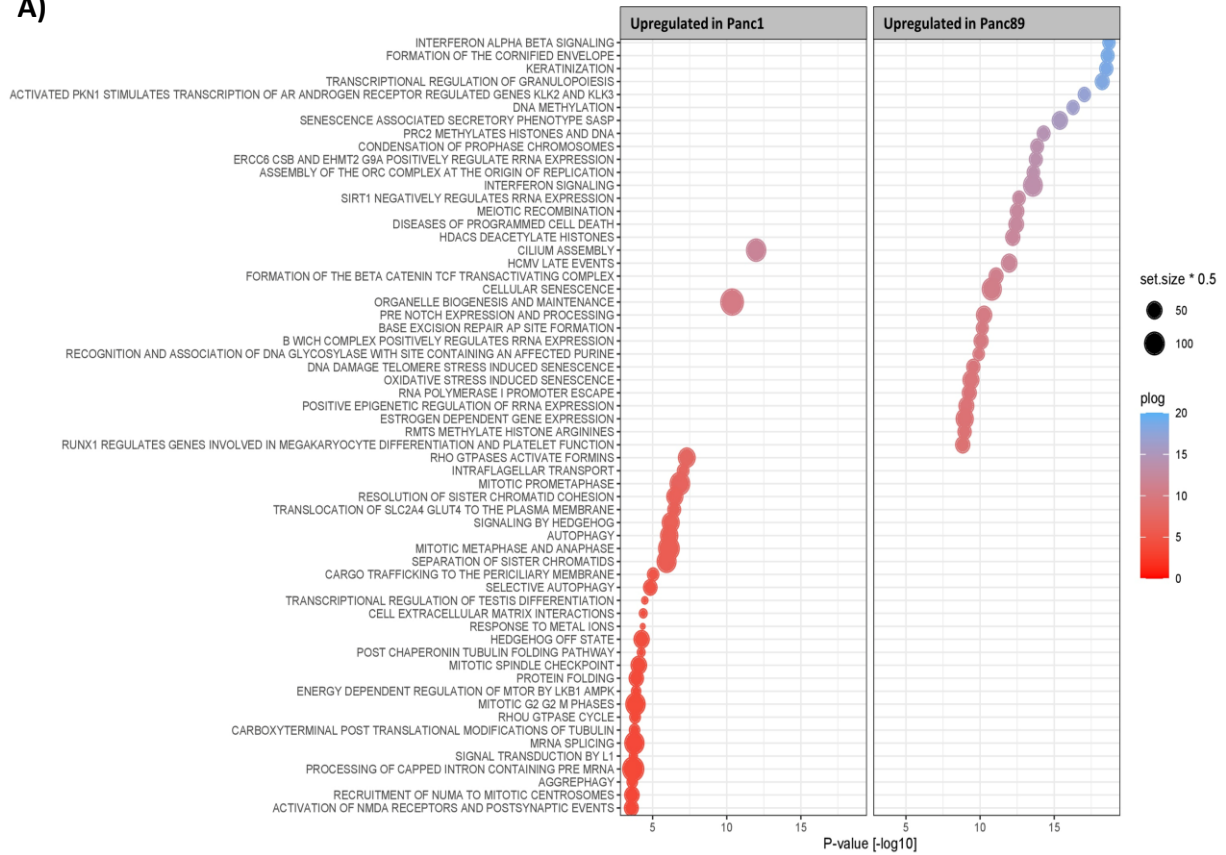
Next, a pathway enrichment of sequenced RNA samples from parental Panc1 and Panc89, Holo- and Paraclone cells was performed in order to evaluate differences in the Reactome and stemness pathway activities. Gene sets from the Molecular Signatures Database collection C2²⁷⁷, selected for Reactome pathways and a stemness gene set “MUELLER PLURINET”, were evaluated *via* Gene Set Enrichment Analysis (GSEA). GSEA is a computational method that evaluates if a previously defined gene set significantly differs between two biological states^{278,279}. For this purpose, in every GSEA two cell variants of the six PDAC cell populations (parental Panc1 and Panc89, Holo- and Paraclone cells) were compared regarding their variation in upregulated or downregulated gene expression. Comparisons were performed between the parental Panc1 and Panc89 cell lines and between the Holo- and Paraclones of each cell population.

GSEAs on the differential effect sizes on the Reactome pathways of parental Panc89 cells indicated a stronger Interferon signaling, DNA methylation, and senescence activity compared to parental Panc1 cells. In contrast, Panc1 cells showed more cilium assembly, transport, autophagy, and mitotic processes (Figure 7 A). Comparative analysis of Panc1 Holo- and Paraclone samples showed Panc1 Holoclone cells to have increased activity of mRNA splicing, DNA repair, chromatin modifying enzymes, transcriptional regulation by TP53, cell cycle checkpoints and Mueller Plurinet, the latter being a strong indicator for stemness properties ²⁸⁰. Panc1 Paraclones, on the other hand, showed increased activity of ECM matrix organization and neutrophil degranulation (Figure 7 B). Panc89 Holoclones showed increased activity of different mitotic phases, cell cycle checkpoints, organelle biogenesis and maintenance, and transcriptional regulation of TP53 and Mueller Plurinet, while Panc89 Paraclones showed increased activity for neutrophil degranulation (Figure 7 C). In summary, parental Panc1 and Panc89 cells showed major differences in their pathway activities, but also between the derived Holo- and Paraclone cells distinct differences could be observed.

GSEA performed on the marker genes for PDAC subtypes according to Moffitt et al. ²⁸ revealed that Panc89 cell variants had gene features related to both the classical-like and basal-like PDAC subtypes, with a stronger tendency towards the basal subgroup, irrespective of the gene classifier size from 25 to 250 genes. In contrast, the expression pattern of Panc1 cell variants could not be clearly assigned to one subtype, but showed a tendency towards a classical-like subtype (Supplementary figure 1). Of note, PDAC cell line models could have mixed characteristics due to their organ of origin and their differentiation status.

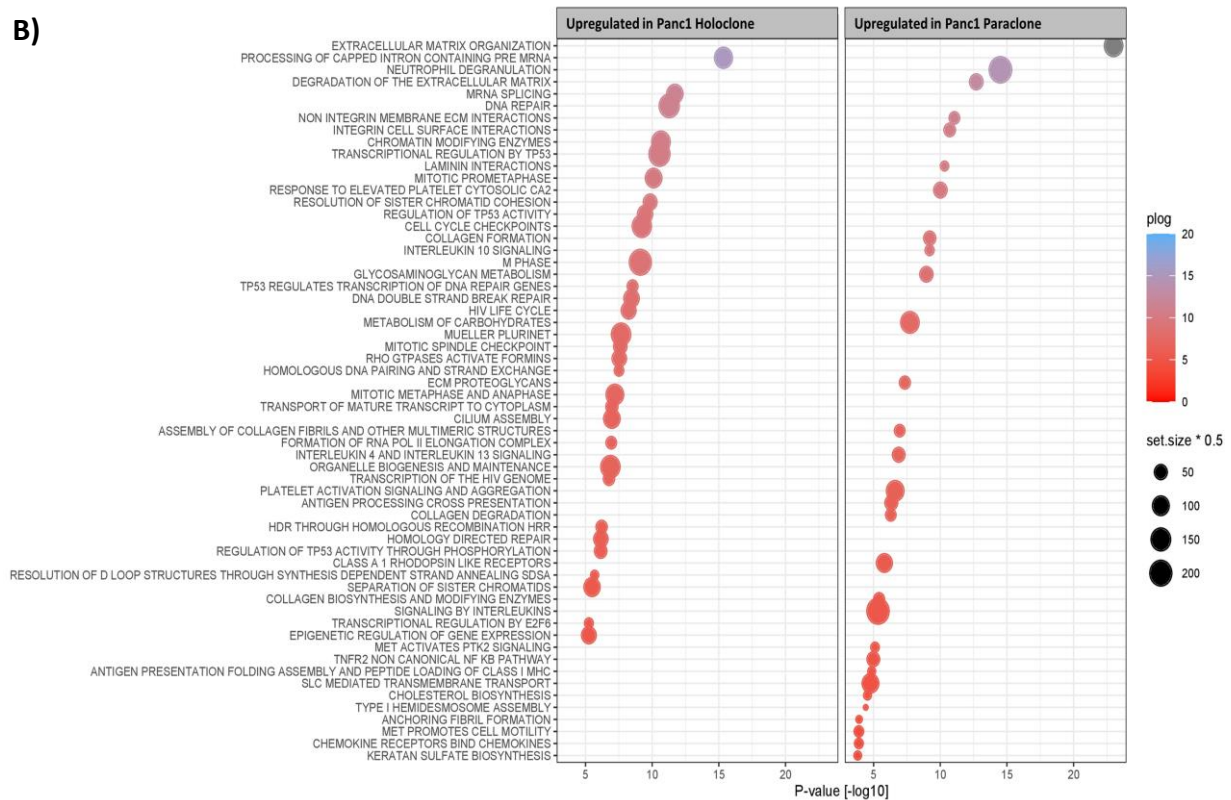
A)

Parental PDAC cells



Panc1 cell clones

B)



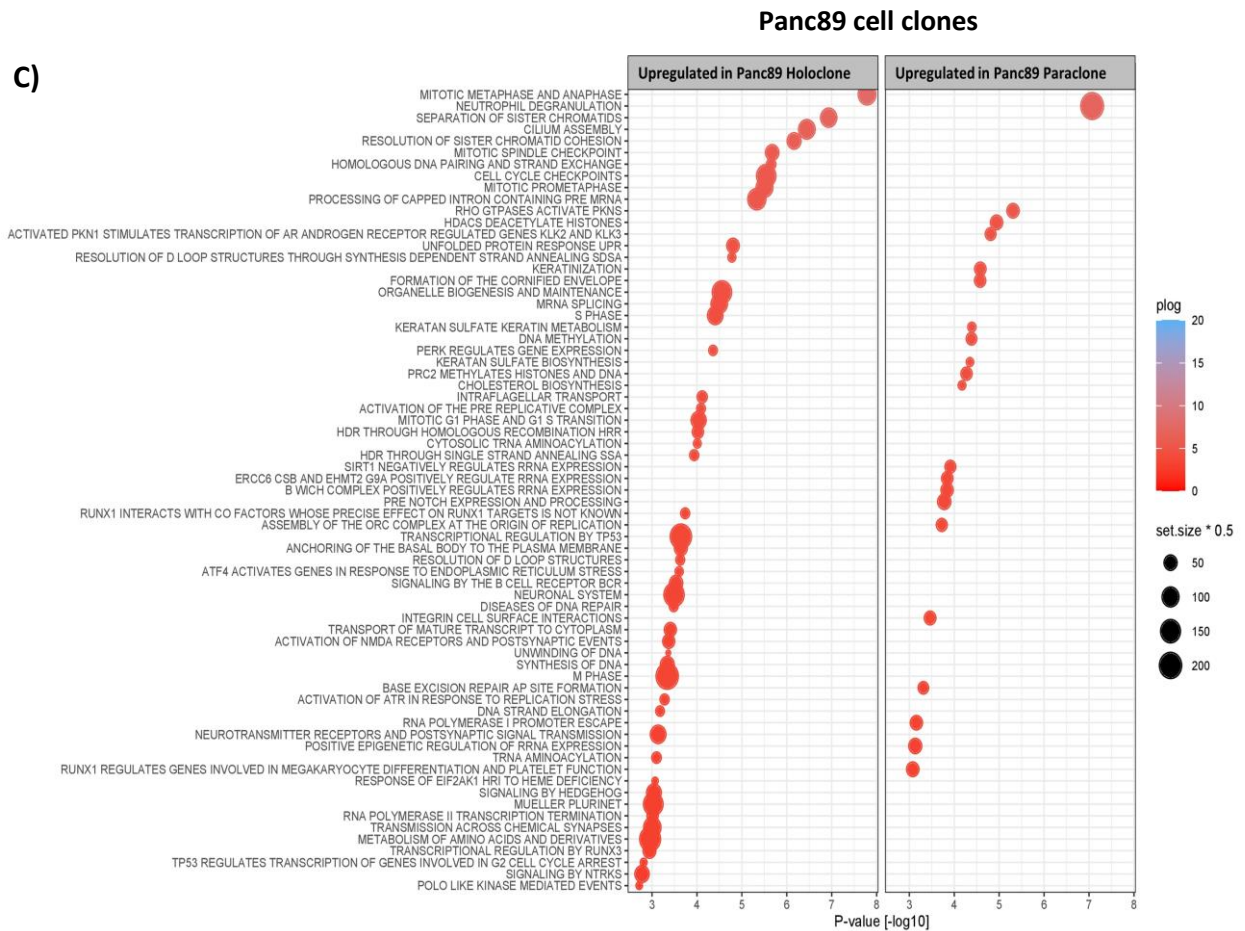


Figure 7 Gene Set Enrichment Analysis on the differential effect sizes on the reactome pathways in parental Panc1 and Panc89 as well as Holo- and Paraclone cell samples. Comparative Gene Set Enrichment Analysis (GSEAs) for A) parental Panc1 cells vs parental Panc89 cells, B) Panc1 Holoclones vs Paraclones and C) Panc89 Holoclones vs Paraclones are shown. GSEA on the shrunken log2 fold changes from apegIrn²⁷⁴ was performed using GAGE (v2.48.0)²⁷⁵ against Hallmark, Reactome and Gene Ontology Biological Processes (GOBP) gene sets extracted from the msigdb R package (v7.5.1). Independent replicates n = 3.

4.2.1.3 Gene Set Variation Analysis of parental Panc1 and Panc89 cells and derived Holo- and Paraclones revealed different regulation patterns of EMT, stemness and invasion

Gene sets that are associated with a positive or negative regulation on the EMT program, stemness and invasion properties²⁸¹ were comparatively analyzed for all Panc1 and Panc89 cell variants *via* Gene Set Variation Analysis (GSVA). GSVA was linked to an activity score to the signaling pathway of every sample, which were then z-transformed with mean = 0 and standard deviation = 1. Comparing Panc1 and Panc89 cells, the data showed positive regulation of EMT and invasion in parental Panc1 cells, while parental Panc89 cells showed a rather negative regulation of EMT. Panc1 cell lines were further characterized by high invasive properties, reflected by upregulation of genes that positively regulate EMT and Invasion (Figure 8).

Further, Panc1 Holoclone cells also showed a trend towards positive EMT regulation (P-value = 0.15), which could not be determined for Panc1 Paraclone cells. For Panc89 cell variants it was found that parental cells showed an overall low but significant increase of positive regulation of invasion compared to Panc89 Holoclones (P-value = 0.026) and Panc89 Paraclones (P-value = 0.0015) (Figure 8). For the analysis of CSC properties for all analyzed cell variants the Mueller Plurinet gene set was used. Comparing the stemness characteristics in Panc1 cell variants it was found that Panc1 Holoclone cells showed the strongest CSC properties, followed by Panc1 parental cells. Panc1 Paraclone cells showed the weakest CSC characteristics of all Panc1 cell variants. In Panc89 cell variants it could be determined that parental Panc89 cells showed significantly higher CSC activity compared to Panc89 Paraclones, while for CSC activity between parental Panc89 cells and Panc89 Holoclone cells as well as Panc89 Holo- and Paraclone cells no significant differences could be observed (Figure 8). Of note, within all Panc1 and Panc89 cell samples, a strong data heterogeneity could be investigated.

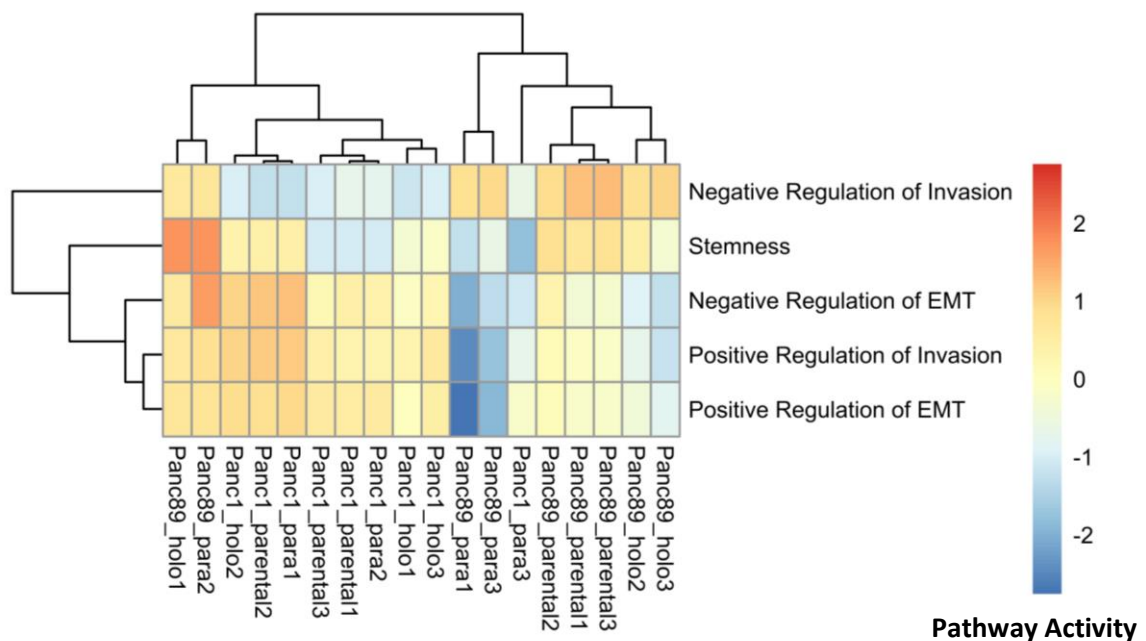


Figure 8 Gene Set Variation Analysis of gene sets associated with positive or negative regulation on the EMT program, stemness and invasion properties in parental Panc1 and Panc89, Holo- and Paraclone cells. Gene sets from CancerSEA²⁸¹ were used to investigate whether Panc1 and Panc89 cell variants showed different activation of genes that are strongly associated with stemness (by Mueller Plurinet gene set), EMT and cell invasion. The numbering of all cell variants represents the number of the independent replicate (independent replicates n = 3). (EMT = Epithelial-to-Mesenchymal-Transition, holo = Holoclone, para = Paraclone)

4.2.1.4 Marker gene analysis of parental Panc1 and Panc89, Holo- and Paraclone cells showed distinct EMT- and CSC-marker profiles

In total, 10,429 out of 17,354 detected genes were found to be differentially regulated between parental Panc1 and Panc89 cell lines (adj. P-value ≤ 0.01), which confirmed the large sample separation in the PCA. Moreover, first EMT and CSC characteristics of analyzed variants from Panc1 and Panc89 cells were determined as exemplified by high expression of *NES*, *VIM*, *L1CAM* and *ZEB1* in Panc1 cells, and *CDH1*, *SOX2* and *OVOL2* in Panc89 cells (Figure 9). In detail, all Panc1 cell variants expressed similar amounts of the mesenchymal markers *VIM* and *L1CAM* and the EMT inducer *ZEB1*. Expression of the CSC-marker *NES* was highest in parental Panc1 cells, followed by Panc1 Holoclones, and lowest in Panc1 Paraclones, the latter with a decreased *NES* expression of about 50% compared to parental Panc1 and Holoclone cells (Figure 9). Contrary, all Panc89 cell variants expressed similar amounts of the epithelial marker *CDH1* (E-cadherin). Furthermore, parental Panc89 and Panc89 Paraclone cells both expressed similar levels of *L1CAM*, while Panc89 Holoclone cells showed only faint *L1CAM* expression. Moreover, Panc89 Paraclones were found to express higher levels of the mesenchymal marker *VIM* and the EMT-inducers *ZEB1* and *ZEB2* compared to parental Panc89 and Holoclone cells. Regarding the expression of CSC-markers it was found that parental Panc89 and Panc89 Paraclone cells expressed similar levels of *OVOL2*, whereas *SOX2* was similarly expressed in both parental Panc89 and Panc89 Holoclone cells (Figure 9).

As clear differences could be determined between the two parental PDAC cell lines but also between their derived Holo- and Paraclones, RNA expression differences should be confirmed by qPCR analysis.

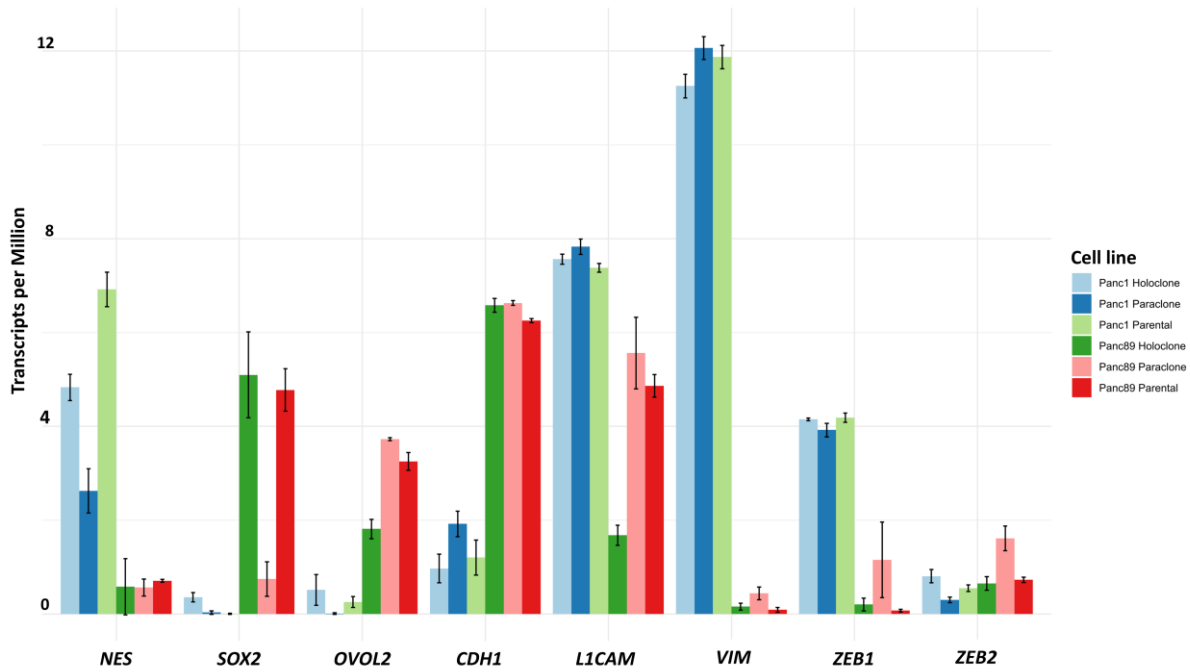


Figure 9 Transcriptomic marker gene analysis of parental Panc1 and Panc89, Holo- and Paraclone cells. Transcriptomic analysis revealed different expression of CSC- and EMT- associated marker genes in parental Panc1 and Panc89 as well as Holo- and Paraclone cells. *NES*, *OVOL2* and *SOX2* represented CSC-marker genes, *CDH1* (epithelial), *VIM* and *L1CAM* (mesenchymal) represented EMT-marker genes and *ZEB1* and *ZEB2* represented marker genes for EMT-induction. Y-axis shows the transcripts per million reads, X-axis shows analyzed genes. Data are represented as means with SD. Independent experiments n = 3. (CSC = Cancer stem cell, EMT = Epithelial-to-Mesenchymal-Transition)

4.2.1.5 qPCR analysis revealed Panc1 Holoclone cells to exhibit a mesenchymal stemness phenotype while an epithelial stemness phenotype characterizes Panc89 Holoclone cells

To confirm the differences regarding the EMT and CSC characteristics identified by RNA sequencing, RNA expression of CSC- and EMT-markers was analyzed in parental as well as Holo- and Paraclone cell variants of Panc1 and Panc89 cells by qPCR. Thus, expression of the CSC-markers *NES* (Nestin), *SOX2* and *OVOL2* (ZNF) was analyzed along with the EMT-markers *CDH1* (E-cadherin), *L1CAM*, *VIM* (Vimentin), *ZEB1* and *ZEB2* and normalized to GAPDH (Glycerinaldehyde-3-phosphate-Dehydrogenase), which was used as reference gene.

Whereas *NES* expression could not be detected in any cell population of Panc89 cells, it was highly expressed in parental Panc1 and Panc1 Holoclone cells. In Panc1 Paraclone cells *NES* expression was detectable, but only in low amounts (Figure 10 A). Contrary, *SOX2* expression was nearly not detectable in all Panc1 cell lines but *SOX2* was highly expressed in Panc89 Holoclone cells, moderately expressed in parental Panc89 cells and nearly not detectable in Panc89

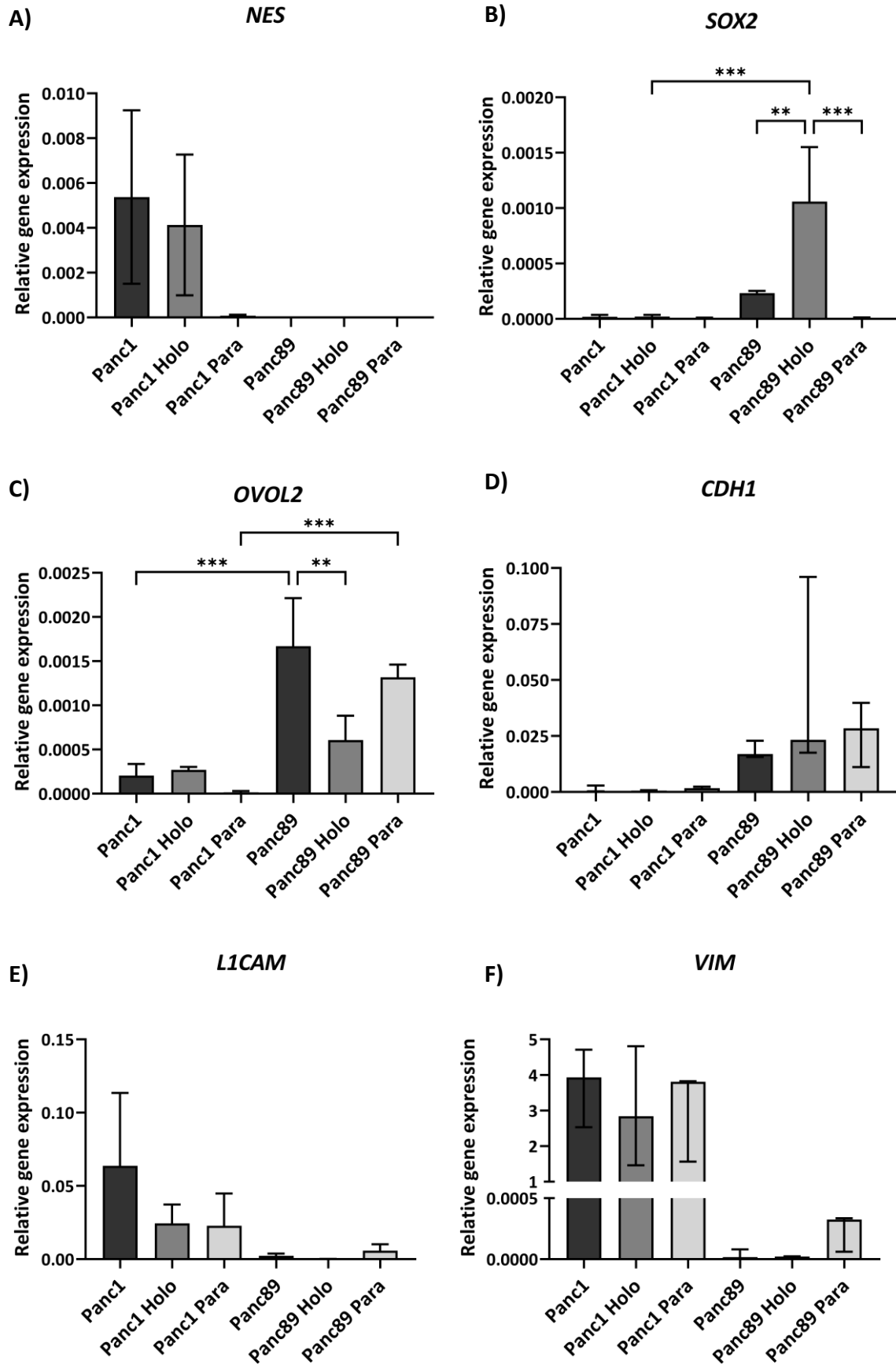
Paraclone cells (Figure 10 B). *OVOL2* was higher expressed in all Panc89 cell variants compared to Panc1 cell variants, but with distinct differences between the clones. It was moderately expressed in parental Panc1 and Holoclone cells, but not in Panc1 Paraclone cells. In contrast, *OVOL2* expression was highest in parental Panc89 cells, followed by Paraclone cells and Panc89 Holoclone cells exhibited the lowest *OVOL2* expression among all Panc89 cell variants but still higher expression compared to any Panc1 cell line (Figure 10 C).

Next, expression of the epithelial marker *CDH1* was analyzed. While *CDH1* expression was almost undetectable in all Panc1 cell variants, it was clearly expressed in all Panc89 cell variants, not showing any significant differences in the expression level (Figure 10 D).

An inverse expression pattern of mesenchymal markers *L1CAM* and *VIM* was detectable in the PDAC cell lines (Figure 10 E, F). All Panc1 cell populations showed higher expression of *L1CAM* and *VIM* compared to Panc89 cell variants, with parental Panc1 cells exhibiting the highest expression of both markers. Compared the Panc89 cell variants, Panc89 Paraclone cells showed the highest expression of either mesenchymal marker compared to parental and Holoclone cells (Figure 10 E, F).

Finally, expression of the two EMT-inducing transcription factors *ZEB1* and *ZEB2* was analyzed. Albeit *ZEB1* expression was generally low in all PDAC cells, it was significantly higher expressed in all Panc1 cell variants compared to Panc89 cell variants (Figure 10 G). While no differences in the expression level could be determined between the three Panc1 cell variants, Panc89 Paraclone cells exhibited higher *ZEB1* expression compared to parental and Holoclone cells. Regarding *ZEB2* expression, highest expression was detectable in Panc1 Holoclone cells followed by expression in Panc89 Paraclone cells. All other cell variants from Panc1 and Panc89 cells exhibited a rather low expression (Figure 10 H).

In summary, qPCR analysis confirmed the expression of EMT- and CSC-marker genes determined in the transcriptomic analysis.



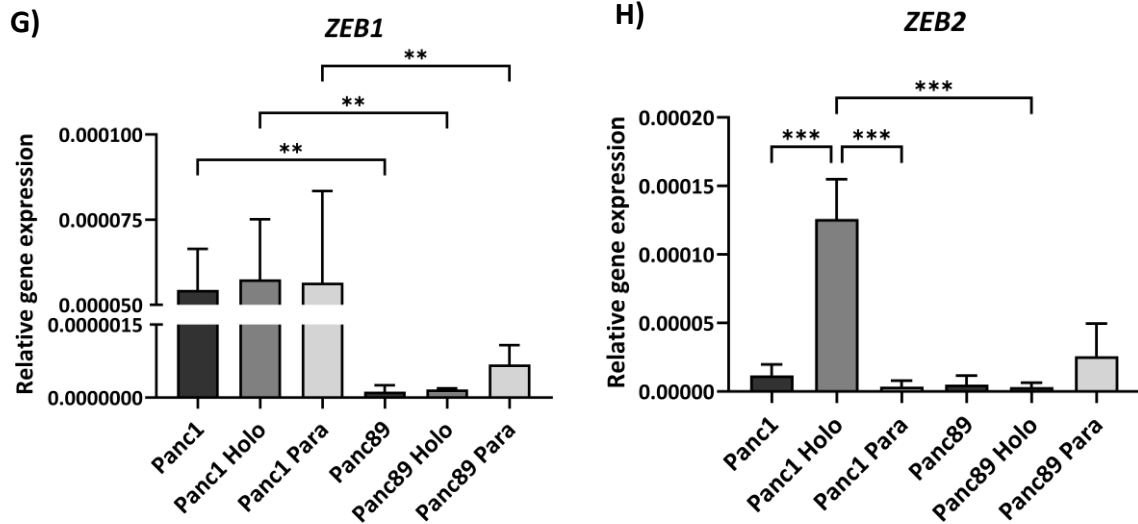


Figure 10 Gene expression analysis of EMT- and CSC-associated genes in parental Panc1 and Panc89, Holo- and Paraclone cells via quantitative real-time polymerase chain reaction. RNA of basal parental Panc1 and Panc89 as well as Holo- and Paraclone cells was isolated and transcribed into cDNA, which was used for quantitative real-time polymerase chain reaction (qPCR) analysis. Expression of the GOI was normalized to the expression of the reference gene GAPDH, and is thus shown as relative gene expression. The stemness marker A) *NES*, B) *SOX2* and C) *OVOL2* were selected as CSC panel. D) *CDH1*, E) *L1CAM* and F) *VIM* were chosen as EMT panel, and G) *ZEB1* and H) *ZEB2* were elected to represent EMT-inducers. Y-axis of *VIM* and *ZEB2* graphs were segmented to provide accurate representation of data. Parametric relative gene expression data were analyzed via one-way ANOVA and Tukey's multiple comparison with data representation as mean with SD. Kruskal-Wallis analysis and Dunn's multiple comparison test was used for nonparametric data and are represented as median with interquartile range. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$, independent replicates $n = 3$. (SD = Standard deviation, Holo = Holoclone, Para = Paraclone)

Overall, these data indicated immense differences in the transcriptomic profiles of PDAC cells originating from primary and secondary tumor sites. While Panc1 cells have presumably already undergone the EMT program and showed a mesenchymal, invasive phenotype, Panc89 cells presented an epithelial non-invasive phenotype. Regarding the CSC populations, both Panc1 and Panc89 Holoclone populations were characterized by activation of similar stemness-associated pathways, underscoring their CSC phenotype. Further, mesenchymal Panc1 Holoclone cells were characterized by pronounced expression of the CSC-marker *NES*, while epithelial Panc89 Holoclone cells were characterized by high *SOX2* expression. Panc1 Paraclones showed no notable CSC-marker gene expression but mesenchymal properties, while Panc89 Paraclone cells were characterized by the expression of a partial/hybrid EMT cell state, indicated by co-expression of epithelial and mesenchymal markers as well as *OVOL2*.

4.2.2 Immunofluorescent staining of EMT- and CSC-markers confirmed gene expression pattern in parental Panc1 and Panc89, Holo- and Paraclone cells

In order to further validate the different EMT and CSC phenotypes of the respective PDAC cell lines, double IFS were performed detecting a CSC- and an EMT-marker which were identified by qPCR analysis to be differentially expressed in the Holo- and Paraclone cells. Since Panc1 Holo- and Paraclone cells showed differences in their *NES* and *ZEB2* expression status, while Panc89 Holo- and Paraclone cells clearly differed regarding their expression of *SOX2* and *L1CAM*, these markers were used for IFS.

The parental Panc1 cell population comprised cells exhibiting both NESTIN and ZEB2 expression but also cells lacking both markers supporting the view of a heterogeneous cell population consisting of CSCs and non-CSCs. In line with the gene expression status, clear differences were determined between Panc1 Holo- and Paraclone cells with Panc1 Holoclone cells being clearly positive for NESTIN and ZEB2, while Panc1 Paraclone cells lacked expression of both markers (Figure 11 A).

Analysis of SOX2 and L1CAM in parental Panc89 cells revealed expression for both markers, but showed no co-expression pattern of SOX2 and L1CAM within the heterogeneous population, as formed colonies were either positive for SOX2 or L1CAM. In contrast, Panc89 Holoclone cells showed SOX2 positive cells which lacked L1CAM expression, while Panc89 Paraclone cells exhibited strong L1CAM expression which were SOX2 negative (Figure 11 B), being well in line with the respective gene expression status.

Overall, these data indicate the existence of different CSC phenotypes which can be associated either with mesenchymal or epithelial characteristics. While Panc1 Holoclone cells are characterized by pronounced expression of the CSC-marker NESTIN along with a mesenchymal phenotype, Panc89 Holoclone cells are characterized by high SOX2 expression exhibiting an epithelial phenotype.

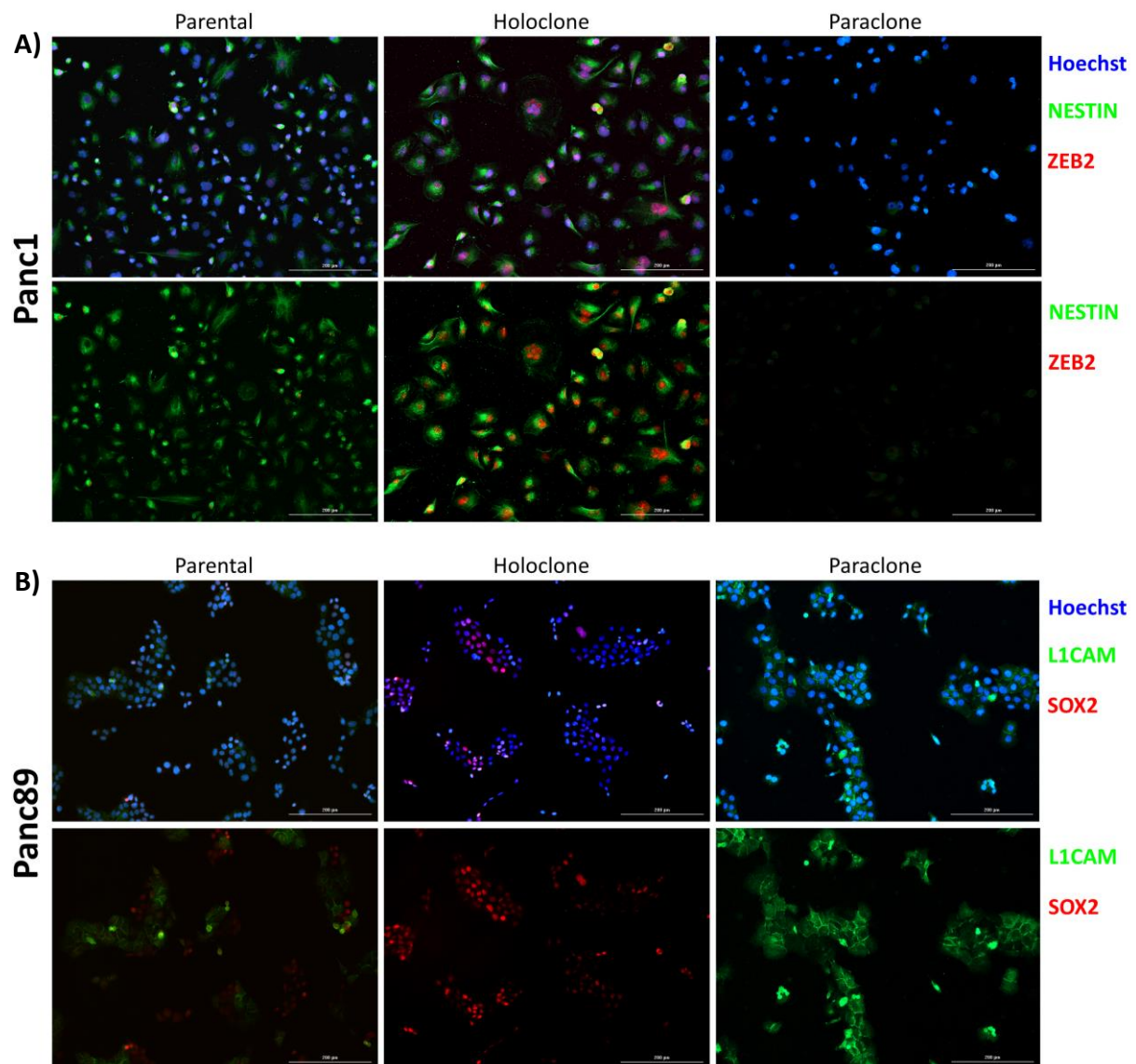


Figure 11 Immunofluorescent staining of EMT- and CSC-markers in parental Panc1 and Panc89, Holo- and Paraclone cells. Cells were seeded on coverslips and for Panc1 cell lines the A) CSC-marker NESTIN and the EMT-inducer ZEB2 were stained, while B) the CSC-marker SOX2 and the mesenchymal EMT-marker L1CAM were stained in Panc89 cell lines. Additionally, all cell nuclei were stained with Hoechst33258 (Hoechst). Representative images from n = 3 independent experiments are shown. Scale bar = 100 μ m.

4.3 Functional analysis of Panc1 and Panc89 cell lines

4.3.1 Panc89 cells showed enhanced cell growth rates compared to Panc1 cells

Having identified different CSC phenotypes, the functional behavior of these CSC phenotypes exhibiting different EMT characteristics in comparison to their parental population and the non-CSC population was analyzed. Thus, we determined the cell growth behavior of parental Panc1 and Panc89 as well as Holo- and Paraclone cell lines by measuring cell confluence and nuclei counts every 24 h over a period of 168 h. Growth rate values of both cell confluence ($k_{\text{Cell confluence [\%]}}$) and nuclei count analysis ($k_{\text{Nuclei count [\#]}}$), represented as k_{Mean} , were determined by the calculation of logistic growth (Table 8).

Table 8 Determination of $k_{\text{Cell confluence [\%]}}$, $k_{\text{Nuclei count [\#]}}$ and respective k_{Mean} values for parental Panc1 and Panc89, Holo- and Paraclone cells (n = 3 independent experiments)

Panc1			
	Parental	Holoclone	Paraclone
$k_{\text{Cell confluence [\%]}}$	0.03033	0.02984	0.02394
$k_{\text{Nuclei count [\#]}}$	0.03476	0.03122	0.02150
k_{Mean}	0.033	0.031	0.023

Panc89			
	Parental	Holoclone	Paraclone
$k_{\text{Cell confluence [\%]}}$	0.05828	0.05387	0.04087
$k_{\text{Nuclei count [\#]}}$	0.05668	0.06170	0.04266
k_{Mean}	0.057	0.058	0.042

Comparing growth rates of parental Panc1 and Panc89 cells it was found that parental Panc89 cells grew 43.4% faster than parental Panc1 cells, with 0.033 k_{Mean} for parental Panc1 and 0.057 k_{Mean} for parental Panc89 cells (Figure 12 E, Table 8). Furthermore, both Panc89 Holo- and Paraclones showed faster cell growth compared to Panc1 Holo- and Paraclone cells (Figure 12 A, B, C, D). Panc89 Holoclone cells showed growth rates of 0.058 k_{Mean} , Panc89 Paraclones cells exhibited 0.042 k_{Mean} (Figure 12 E, Table 8). Panc1 cell clones on the other hand showed decreased growth speed with 0.031 k_{Mean} for Panc1 Holoclones and 0.023 k_{Mean} for Panc1 Paraclones, which represented a decreased growth rate of 47.2% for Panc1 Holoclones

compared to Panc89 Holoclones and a decreased growth rate of 45.6% for Panc1 Paraclone cells compared to Panc89 Paraclone cells (Figure 12 E, Table 8). Moreover, Holoclone cells of either cell line grew faster compared to the respective Paraclone cells. Growth of Panc1 Holoclones was increased by 25.6% compared to Panc1 Paraclone cells, Panc89 Holoclone cells grew 27.7% faster than Panc89 Paraclones, reflecting the fast proliferative character of transit amplifying progenitor cells, arising from asymmetric division of CSC populations, compared to more differentiated cell fractions.

Finally, parental Panc1 and Panc89 cells grew faster than their Holo- and Paraclone cell variants, except for parental Panc89 and Panc89 Holoclone cells which showed approximately the same growth rates. Growth rate of Panc1 Holoclone cells was decreased by 6.2%, growth rate of Panc1 Paraclones was reduced by even 30.25% compared to parental Panc1 cells (Figure 12 E, Table 8). Panc89 Paraclone cells showed decreased growth rate of 27.7% compared to respective parental cells, whereas growth rates of parental Panc89 and Holoclone cells were comparable.

Overall, these data underscore that mesenchymal PDAC cells exhibit a slowly growing phenotype compared to epithelial PDAC cells which also applies to their CSC-fraction.

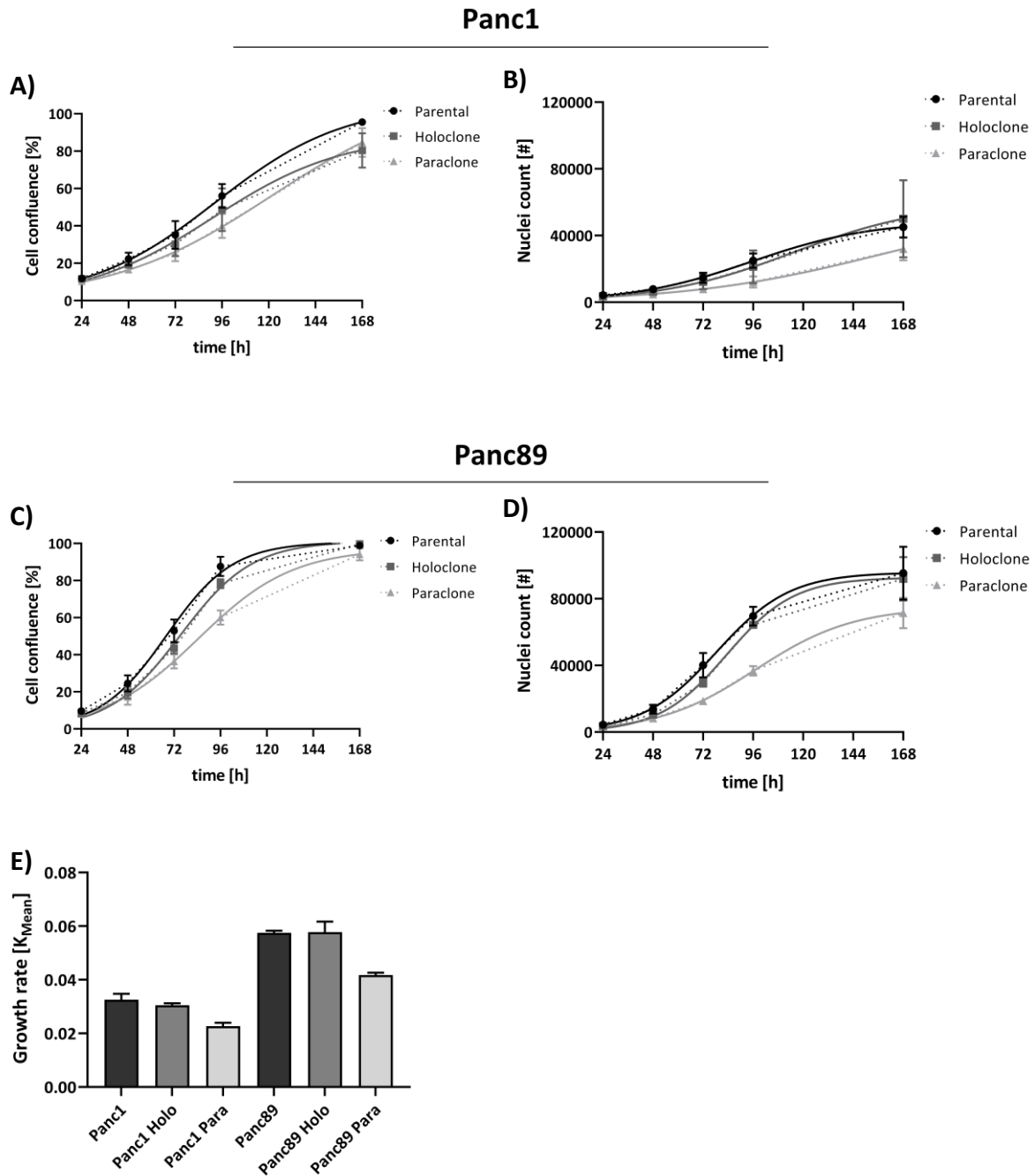


Figure 12 Growth rate analysis of parental Panc1 and Panc89, as well as Holo- and Paraclone cells revealed Panc89 cell lines to show faster cell growth compared to Panc1 cell lines. 0.5×10^4 cells of each cell line were seeded in a 96-well plate in triplicates and cell confluence as well as nuclei number were monitored for 168 h. Logistic growth rate analysis *via* determination of cell confluence for A, C) parental Panc1 and Panc89, Holo- and paraclone cells and logistic growth rate analysis *via* determination of nuclei count for B, D) parental Panc1 and Panc89, Holo- and Paraclone cells are shown. Data are depicted as percent [%] cell confluence and total number [#] of nuclei. Analysis of k for cell confluence and nuclei count was performed by logistic growth analysis *via* Nonlin-fit in GraphPad Prism. E) Mean growth rates calculated from cell confluence and nuclei count analysis. Graphs present mean with SEM of $n = 3$ independent experiments.

4.3.2 Panc1 and Panc89 cell clones essentially differ with respect to survival, tumor growth and metastatic capacity *in vivo*

Finally, to assess whether the identified CSC/EMT phenotypes, which are associated with clearly different functional phenotypes, show differences in their metastatic behavior, a tumorigenicity assay with intrasplenic inoculation of either Panc1 or Panc89 Holo- or Paraclone cells was performed. This assay was already conducted for Panc1 Holo- and Paraclone cells¹⁸⁴, so that the metastatic behavior of Panc89 Holo- and Paraclone cells was assessed accordingly. First of all, the median survival of the differentially inoculated mice was determined. As shown in Figure 13, animals inoculated with Panc89 Holo- or Paraclone cells showed much shorter survival than animals inoculated with Panc1 Holo- or Paraclone cells. The median survival of mice inoculated with Panc89 Holoclone cells was 74 days and 66 days for Panc89 Paraclone cells. In comparison, animals inoculated either with Panc1 Holo- or Paraclone cells showed about twice as long median survival of 146 days (Figure 13). Of note, animals inoculated with Panc1 cell variants were not killed because of the reduced health status due to the tumor burden, rather than that tumor progression was quite slow so that the experiment was terminated. Overall, statistical analysis using Mantel-Cox log-rank test resulted in significantly different survival curves with a P-value of ≤ 0.001 (***).

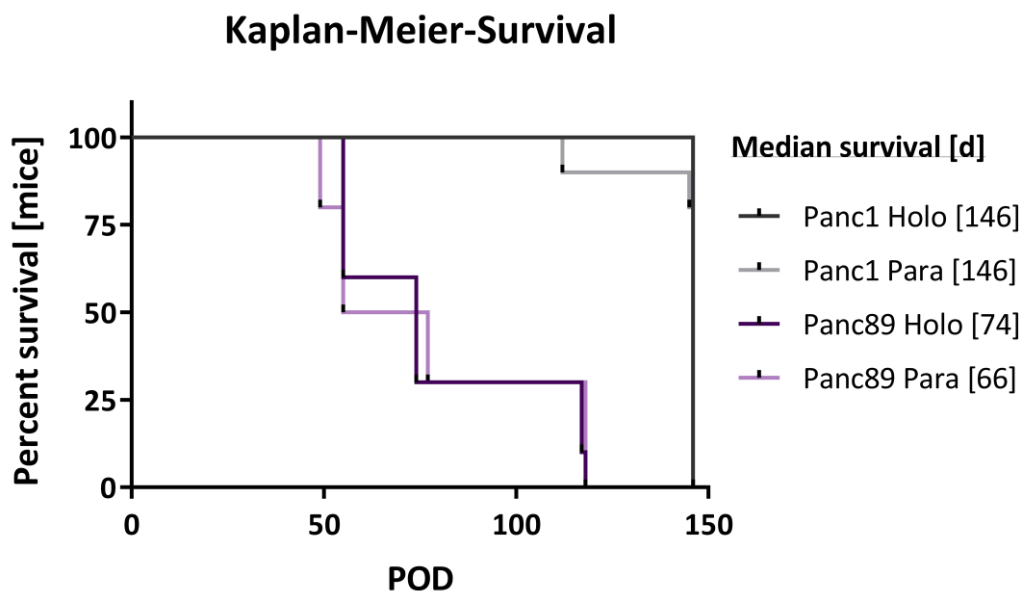


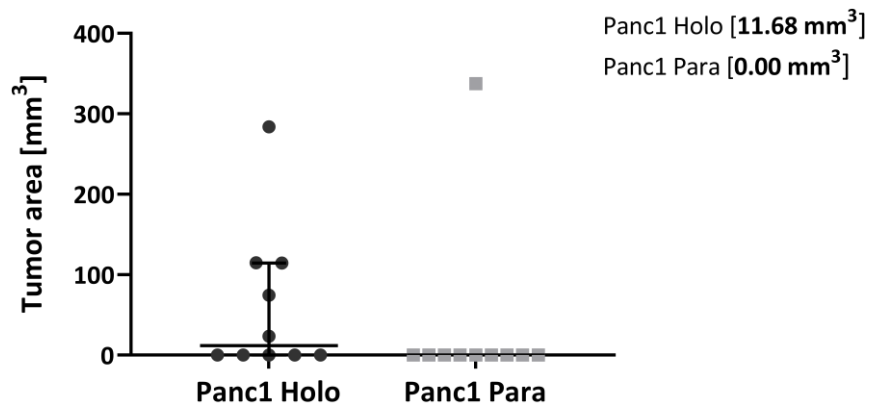
Figure 13 Shorter median survival of animals inoculated with Panc89 Holo- or Paraclone cells. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line). Y-axis shows the percent survival of mice, X-axis presents the POD when the animals were sacrificed because of reduced health status due to tumor burden or to terminate the experiment. Statistical analysis was performed

by Kaplan-Meier-Survival analysis with curve comparison and Mantel-Cox log-rank test, resulting in significantly different survival curves with a P-value of ≤ 0.001 (***) (Holo = Holoclone cells, Para = Paraclone cells, POD = post-operative day)

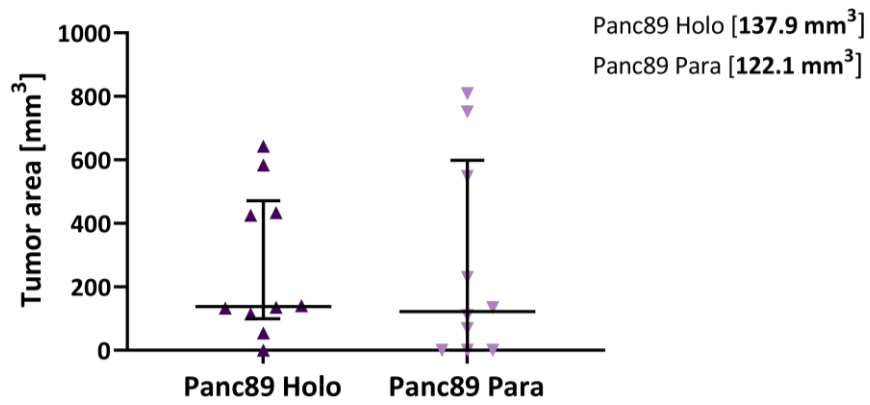
Examination of the macroscopic tumor manifestation revealed clear differences between Panc1 and Panc89 cell variants but also between Holo- and Paraclone cells. Ultrasound examination of living animals revealed macroscopic tumor formation in 7/10 animals with a median tumor area of 11.68 mm³ for Panc1 Holoclone tumors but only in 2/10 animals for Panc1 Paraclones (with no median macroscopic tumor area detectable because one of two tumors could not be measured *via* ultrasound). Contrary, in 10/10 animals inoculated with Panc89 Holoclone cells tumors could be detected with a median tumor area of 137.9 mm³ and in 7/10 animals with a median macroscopic tumor area of 122.1 mm³ for Panc89 Paraclone tumors (Figure 14 A, B). Thus, the shorter survival times of animals inoculated with Panc89 cell variants was associated higher tumor burden compared to Panc1 Holo- and Paraclone cells.

Moreover, Panc1 Holo- and Paraclone tumors were predominantly found in the pancreas, but also in liver and lung, while Panc89 Holo- and Paraclone tumors were predominantly detectable in the peritoneum, followed by pancreas, liver and lung (Figure 14 C, D). In detail, intrasplenic inoculation of Panc1 Holoclone cells led to clearly more tumors compared to Panc1 Paraclone tumors, which were predominantly formed in the pancreas (5/10 animals) and liver (3/10 animals), while inoculation of Panc1 Paraclone cells only led to tumors in pancreas and lung of 1/10 animals (Figure 14). Macroscopically, inoculation of Panc89 Holoclones also led to a higher number of tumors compared to Panc89 Paraclone cells and also compared to all Panc1 cell variants. Panc89 Holoclone tumors were found in the peritoneum of 8/10 animals, in the pancreas of 2/10 animals and in the liver and lung of 1/10 animals. Panc89 Paraclone tumors were found in the peritoneum of 4/10 animals, in the pancreas of 2/10 animals and in the liver of 1/10 animals (Figure 14).

A) **Macroscopic tumor area**



B) Macroscopic tumor area



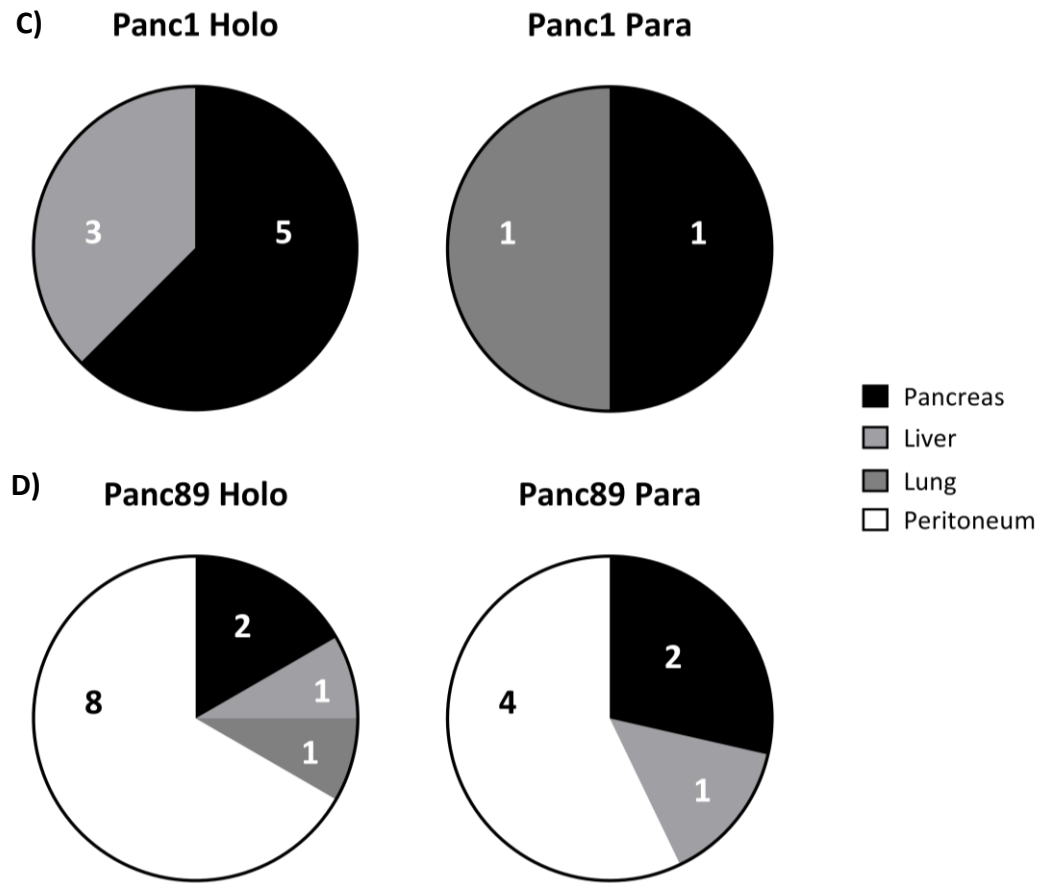
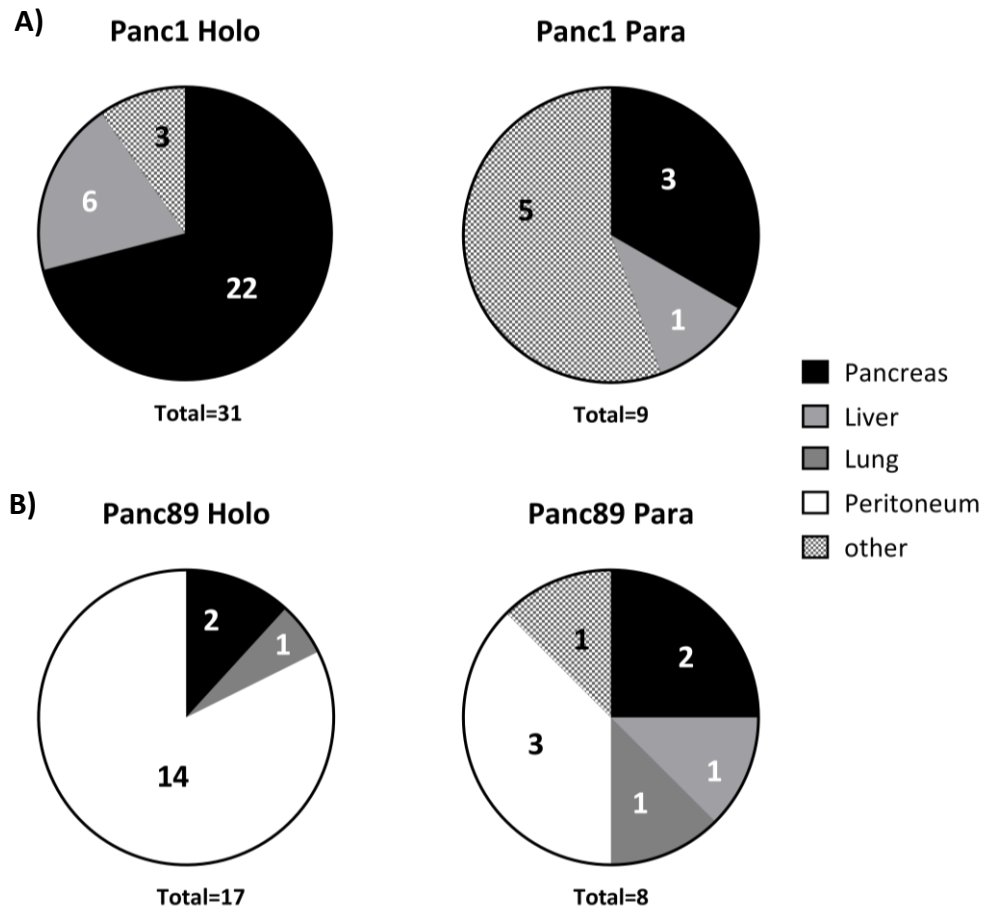


Figure 14 Macroscopic tumor areas and organs of macroscopic tumor manifestation in Panc1 or Panc89 Holo- or Paraclone cell inoculated mice. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line). Organs and tumor formation were monitored *via* ultrasound until experiment termination. A, B) macroscopic tumor areas [mm³] of Panc1 or Panc89 Holo- or Paraclone tumors as well as C, D) number of macroscopic tumor formation in different organs is shown. Statistical analysis was performed using A, B) unpaired t-test for non-parametric data and Mann-Whitney test and C, D) parts of a whole analysis. (Holo = Holoclone cells, Para = Paraclone cells)

Having determined clear macroscopical differences regarding the frequency and site of metastases of the differentially inoculated animals, pancreatic, liver, lung and peritoneal tissues were stained for human PanCK to assess the microscopical metastatic burden in terms of number and size of formed microscopic tumors. While intrasplenic inoculation of Panc1 Holoclone cells led to a total of 31 microscopic lesions in 9/10 animals, only 9 microscopic tumors were found in 8/10 animals inoculated with Panc1 Paraclone cells. Of note, the highest number of microscopic Panc1 Holoclone tumors were found in the pancreas (22/31), while Panc1 Paraclone lesions were mostly found at other sites (e.g., brown fat tissue, duodenum, undefinable in 5/9 metastatic lesions) (Figure 15 A). With 17 microscopic tumors in 10/10 mice the number of microscopic Panc89 Holoclone tumors was much lower compared to Panc1 Holoclones, and

organ manifestation was also very different as most tumors were found in the peritoneum (14/17 metastatic lesions) like observed for the macroscopic tumors (Figure 15 B). The number of animals with Panc89 Paraclone tumors and the total number of microscopic lesions were similar to Panc1 Paraclone cells. Inoculation of Panc89 Paraclone cells gave rise to 8 microscopic tumors in 7/10 animals (Figure 15 B). Here, a slightly higher prevalence of tumor formation in the peritoneum compared to other sites was detectable (3/8 lesions).



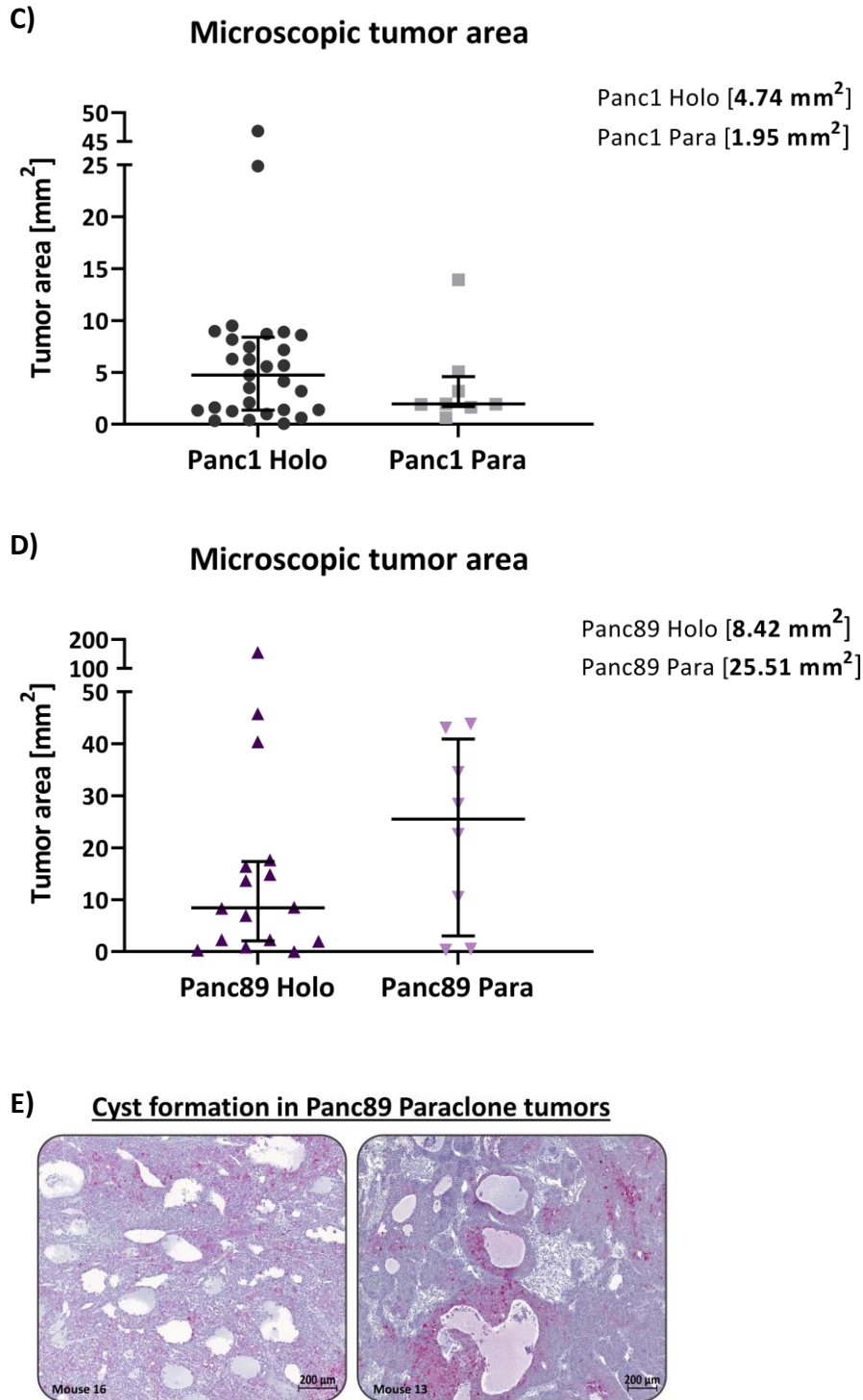


Figure 15 *Post mortem* analysis of microscopic tumors and microscopic tumor area in tissues of Panc1 or Panc89 Holo- or Paraclone cell inoculated animals. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line). Organs and tumor formation of stained tissue sections were microscopically analyzed regarding number and location of microscopic tumors as well as microscopic tumor areas [mm²]. A, B) organs of microscopic tumor formation are represented as parts of a whole. C, D) analysis of microscopic tumor areas of Panc1 or Panc89 Holo- or Paraclone tumors was performed with unpaired t-test for non-parametric data and Mann-Whitney test. E) shows representative images of cyst formation in two independent tumors formed by Panc89 Paraclone cells. (Other = brown fat tissue, duodenum, undefinable; Holo = Holoclone cells, Para = Paraclone cells)

Furthermore, the size of the microscopic tumors was determined by measuring the PanCK positive areas in the tissues. In line with the size of the macroscopic tumors, the median tumor areas of both Panc89 Holo- and Paraclone tumors were larger than Panc1 Holo- and Paraclone tumors (Figure 15 C, D). However, inoculation of Panc89 Paraclone cells led to the largest microscopic tumors with a median tumor area of about 25 mm², followed by Panc89 Holoclone tumors with a median tumor area of about 8 mm² (Figure 15 D). In contrast, Panc1 Holo- and Paraclone cells formed tumors with a median tumor area of less than 5 mm² (Figure 15 C). Besides it could be noted that 5/8 Panc89 Paraclone tumors were characterized by a pronounced cyst formation (Figure 15 E).

Overall, inoculation of Panc89 Holo- and Paraclone cells led to a more pronounced metastatic tumor burden in terms of larger metastases which predominantly manifested in the peritoneum compared to Panc1 Holo- and Paraclone cells which led to smaller tumors mostly in the pancreas. Higher tumor burden after inoculation of Panc89 Holo- and Paraclone cells was associated with significantly shorter survival times. However, particularly the inoculation of Panc1 Holoclone cells led to the highest total number of tumoral lesions being in line with the more pronounced invasive phenotype of the cells (data not shown, doctoral thesis Arne Surrow). The fact that Panc89 Paraclone cells also led to a pronounced tumor burden which was not observed for the two Panc1 cell variants underscores a higher plasticity of these cells. Altogether, these findings indicate clear differences in the metastatic capacity between mesenchymal and epithelial CSCs as well as partial/hybrid cell populations.

4.3.3 Differences in EMT and CSC characteristics of Panc1 and Panc89 Holo- and Paraclone cells verified by immunohistochemical staining of Paraffin-embedded tissue sections

To verify the *in vitro* identified CSC/EMT phenotypes of Panc1 and Panc89 Holo- and Paraclone cells *in vivo*, immunohistochemical staining (IHC) of resected tissues were performed to analyze the expression of the CSC-markers Nestin and SOX2 as well as the EMT-markers E-cadherin, L1CAM, Vimentin, ZEB1 and ZEB2. To clearly identify human PDAC cells, staining of human PanCK was performed. PanCK staining of tumors revealed different morphologies of Panc1 and Panc89 Holo- and Paraclone tumors. While Panc1 Holo- and Paraclone tumors were characterized by scattered arrangement of single tumor cells, Panc89 Holo and Paraclone tumors showed large densely packed cell clusters. Especially Panc89 Holoclone tumors presented distinct PanCK positive cell clusters, surrounded by tumor stroma. Cell clusters in

Panc89 Paraclone tumors were larger, but with a less intense PanCK staining, and also infiltrated with huge tumor stromal areas (Figure 16).

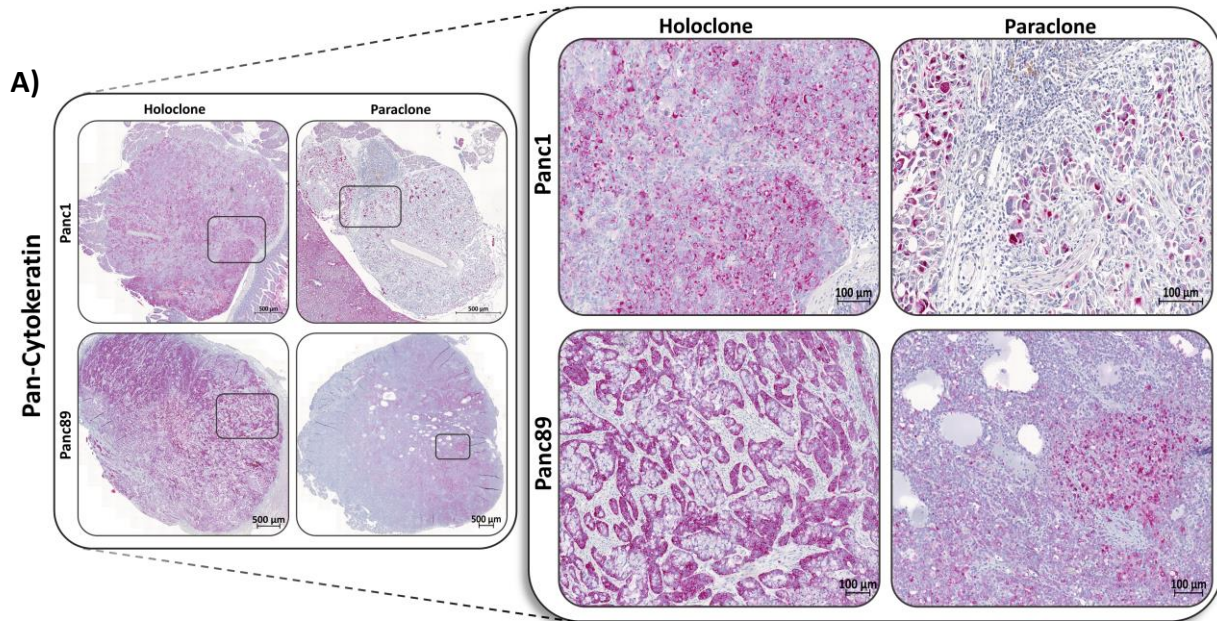


Figure 16 Immunohistochemical analysis of Pan-Cytokeratin in tumor lesions of Panc1 or Panc89 Holo- or Paraclone cell inoculated mice. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line) and extracted tissues and tumor lesions were stained for Pan-Cytokeratin. Scale bars: left = 500 µm; right = 100 µm.

To quantify IHC staining of each marker, a tumor scoring system was developed by determining the amount of positively stained cells (Frequency) and staining intensity (Intensity). Frequency score was set from 1-4 and Intensity score was set from 1-3 (3.4.2, Table 7, Figure 17).

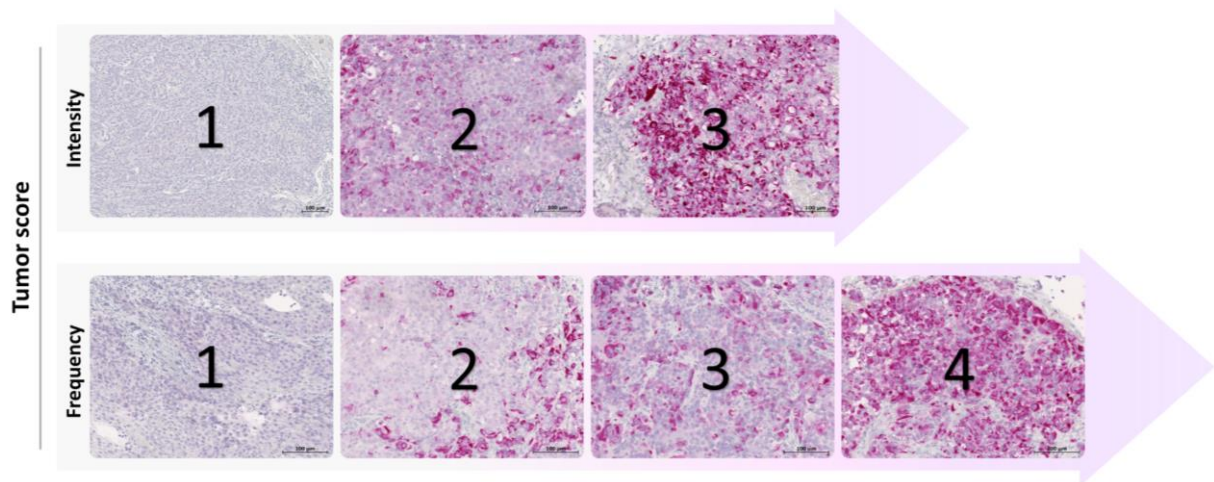
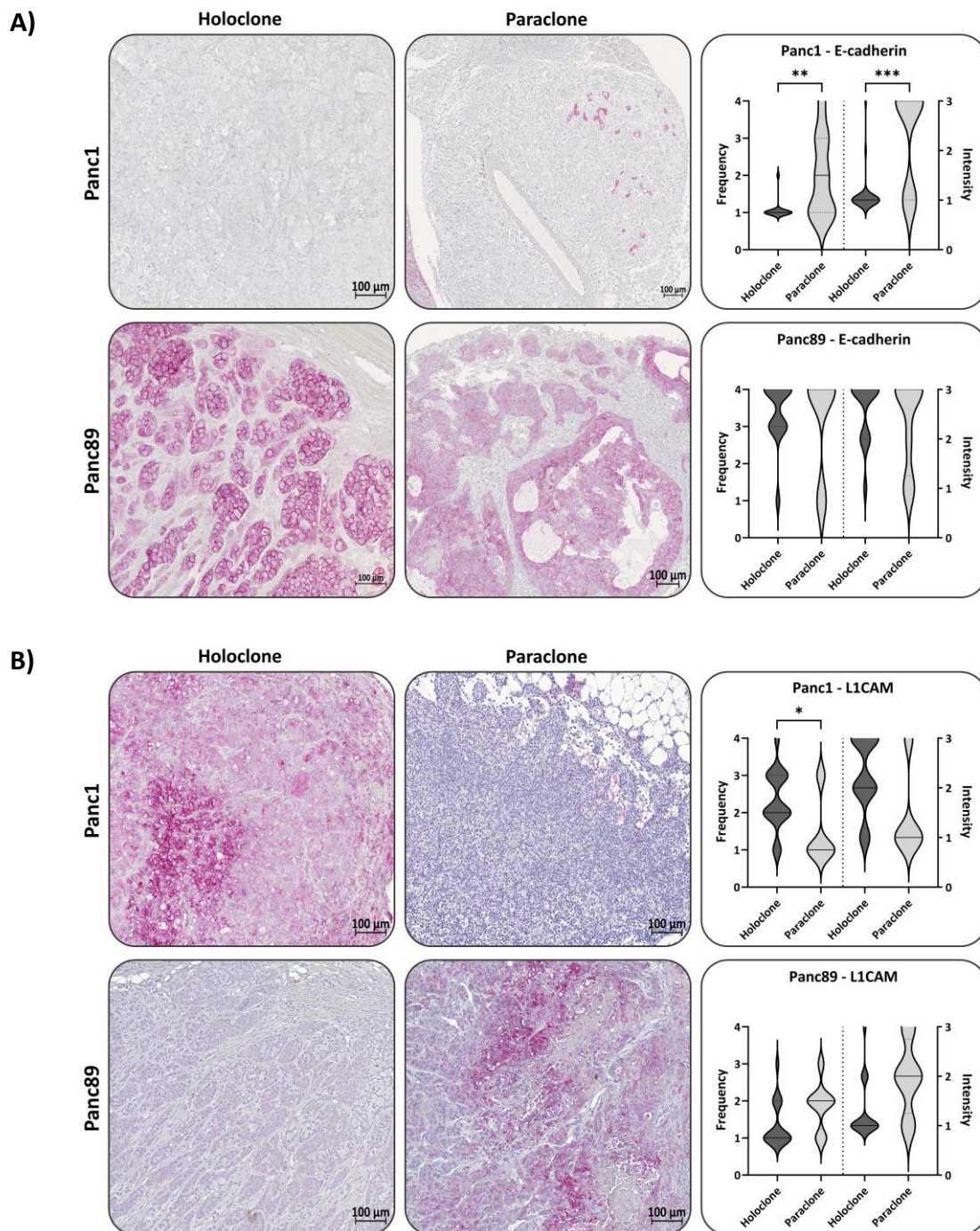


Figure 17 Definition of tumor score by frequency (number of positive cells) and intensity (intensity of positively stained cells) for quantification of immunohistochemical staining of tumor sections from Panc1 and Panc89 Holo- and Paraclone cells *in vivo*. Frequency score was set from 1-4, intensity Score was set from 1-3. The figure shows exemplary pictures of stained tumor tissues for frequency- and intensity score definition. Scale bar = 100 μ m.

Confirming the gene expression data, Panc1 Holoclone cells showed negative to low expression of E-cadherin in analyzed tissues with a Frequency and Intensity score of around 1. Panc1 Paraclone tumors showed a slight but significant increase of E-cadherin expression with a Frequency and Intensity score of 2 compared to Panc1 Holoclone tumor tissues (Figure 18 A). In contrast, in Panc89 Holo- and Paraclone tumors E-cadherin was highly expressed (Frequency and Intensity score of 4) with no considerable differences between Holoclone and Paraclone tumors (Figure 18 A).

L1CAM expression of Panc1 Holoclone tumors was scored with a Frequency of around 2 (Intensity score 1), while Panc1 Paraclone cells showed a significantly lower L1CAM expression (Frequency and Intensity score 1, Figure 18 B). In contrast, Panc89 Holoclone tumors showed a very faint expression of L1CAM (Frequency and Intensity score of 1) compared to Panc89 Paraclone tumors, exhibiting the highest L1CAM expression of all tumors analyzed (Frequency and Intensity score of 3 and 2) (Figure 18 B). However, in some Panc89 Holoclone tumors, L1CAM expression could also be noted (Figure 21). Vimentin was exclusively expressed in

Panc1 Holoclone tumors with a Frequency score of 3 and an Intensity score of 4. Panc1 Paraclone tumors expressed only low amounts of Vimentin (Frequency score 1) but with an Intensity score distribution of 1-3 within the positive areas. Panc89 Holoclone tumors showed no Vimentin expression, and in Panc89 Paraclone tumors the staining of Vimentin was also absent (Figure 18 C) which was not in line with the gene expression analysis, where *VIM* expression could be verified.



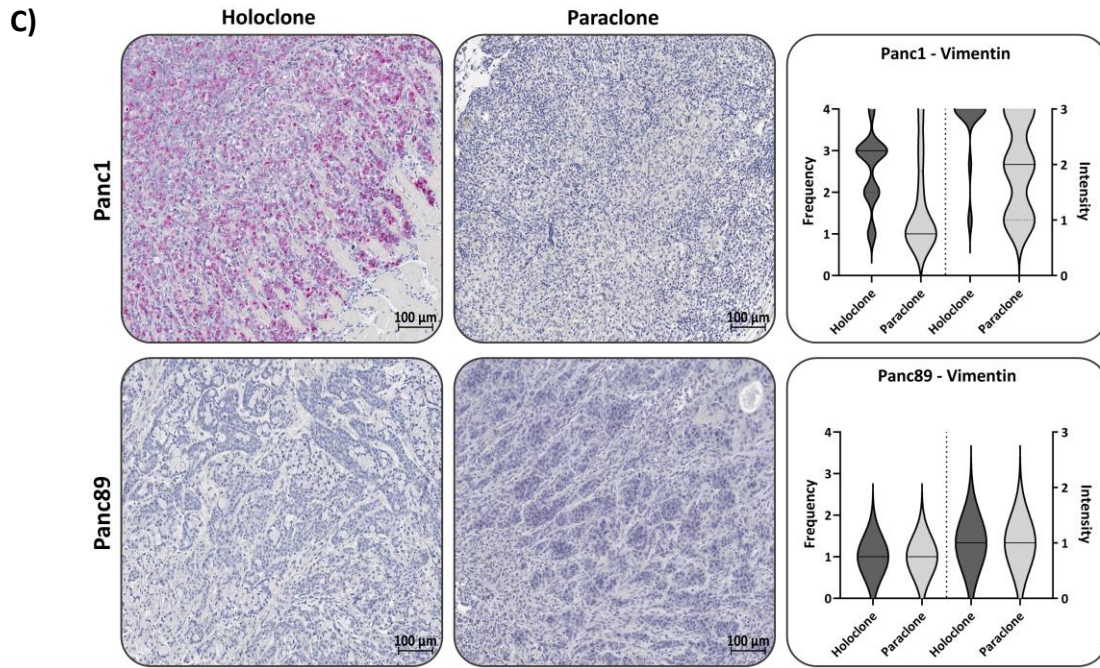


Figure 18 Immunohistochemical analysis of EMT-markers in tumor lesions of mice inoculated with Panc1 or Panc89 Holo- or Paraclone cells. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line) and extracted tissues and tumor lesions were stained for the epithelial marker A) E-cadherin and the mesenchymal markers B) L1CAM and C) Vimentin. Representative pictures of stained tissues of Panc1 and Panc89 Holo- or Paraclone tumors are presented in the left and middle picture panels, the right panel shows the statistical analysis of Frequency and Intensity score of positively stained cells. The upper row shows the data of analyzed Panc1 Holo- and Paraclone lesions, the lower panel shows the data of Panc89 Holo- and Paraclone lesions. Statistical analysis was performed by Shapiro-Wilk normality test, followed by ordinary one-way ANOVA plus Tukey's multiple comparison test for parametric data and Kruskal-Wallis test plus Dunn's multiple comparison test for nonparametric data. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$. Scale bar = 100 µm.

IHC analysis of ZEB1 expression in Panc1 and Panc89 Holo- and Paraclone tumors revealed that Panc1 Holoclone tumors exhibited strong ZEB1 expression (Frequency and Intensity score 4), while Panc1 Paraclone tumors were characterized by low to negative ZEB1 expression with both Frequency and Intensity score of about 1 (Figure 19 A), which was not in line with the ZEB1 gene expression of the cells *in vitro* demonstrating similar RNA levels of ZEB1 in both Panc1 cell variants. In both Panc89 Holo- and Paraclone tumors high ZEB1 expression with a Frequency and Intensity score of 3-4 was noted (Figure 19 A), which also differed from absent ZEB1 gene expression in Panc89 Holo- and Paraclone cells *in vitro*. Of note, ZEB1 and ZEB2 expression were mostly located in the nuclei indicative for their functional activity, and both markers could not only be detected in PDAC cells but also in stroma cells. Panc1 Holoclone tumors showed a significantly higher expression of ZEB2 (Frequency score of 2-3 and

Intensity score of 1) compared to Panc1 Paraclone tumors exhibiting no or only weak expression of ZEB2 (Frequency and Intensity score of 1, Figure 19 B). Panc89 Holoclone tumors showed a slightly lower ZEB2 expression (Frequency and Intensity score 3/2) compared to Panc89 Paraclone tumors with a Frequency and Intensity score of 4/3 (Figure 19 B).

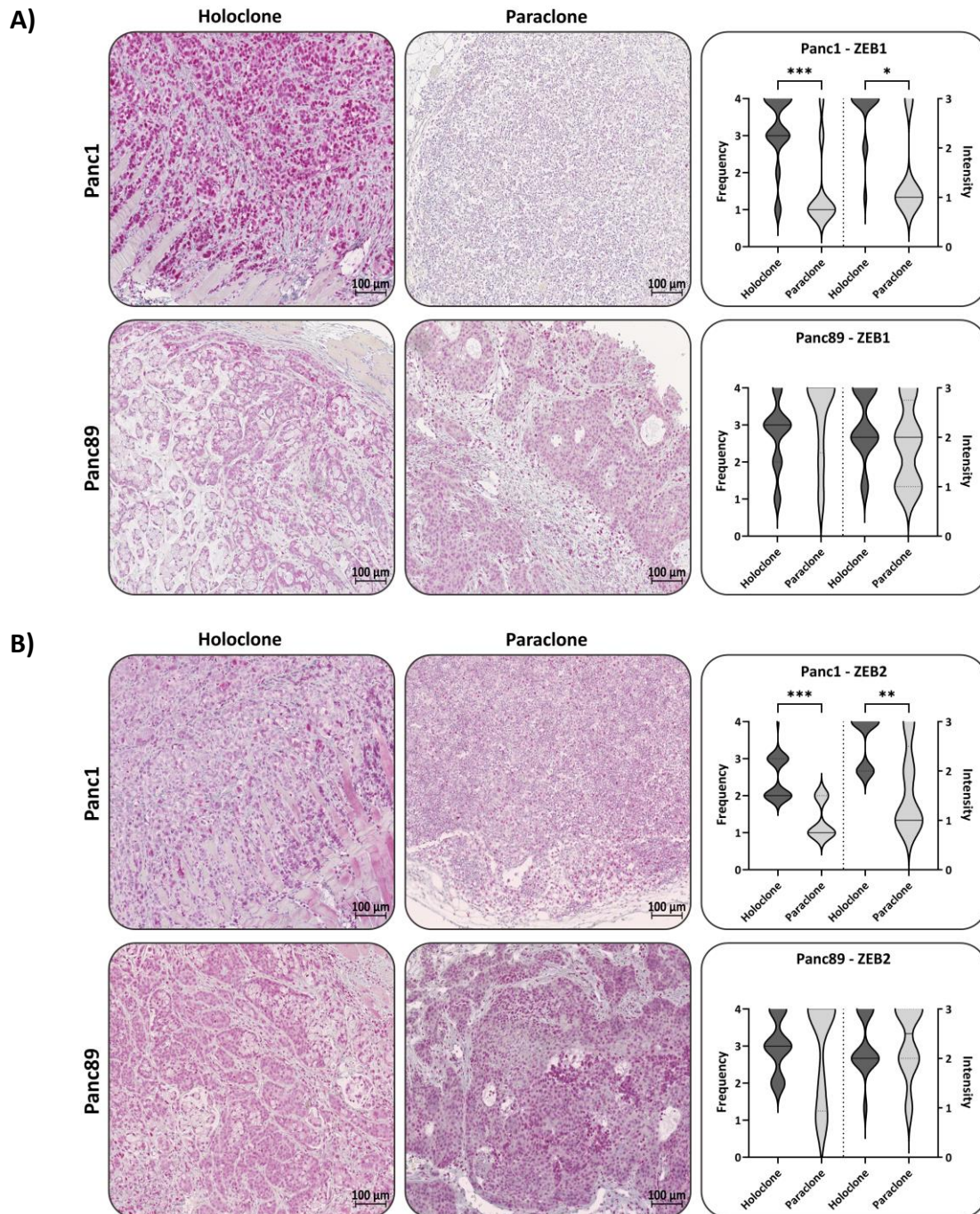


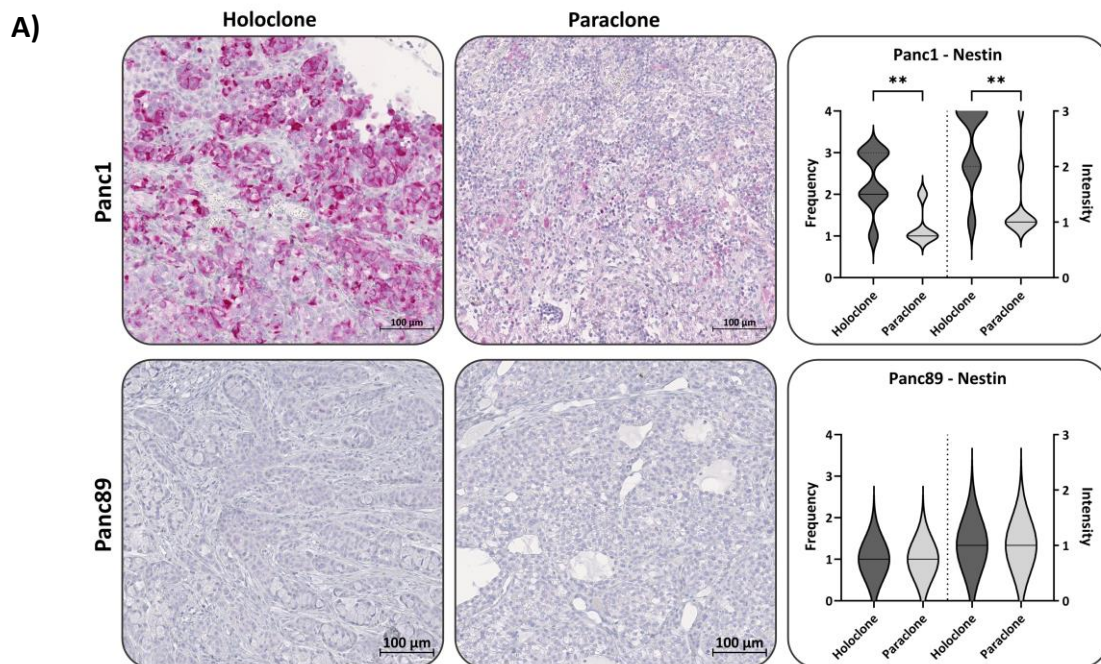
Figure 19 Immunohistochemical analysis of EMT-inducers in tumor lesions of mice inoculated with Panc1 or Panc89 Holo- or Paraclone cells. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line) and extracted tissues and tumor lesions were stained for the EMT-inducers A) ZEB1 and B) ZEB2. Representative pictures of stained tissues of Panc1 and Panc89 Holo- or

- Results -

Paraclone tumors are presented in the left and middle picture panels, the right panel shows the statistical analysis of Frequency and Intensity score of positively stained cells. The upper row shows the data of analyzed Panc1 Holo- and Paraclone lesions, the lower panel shows the data of Panc89 Holo- and Paraclone lesions. Statistical analysis was performed by Shapiro-Wilk normality test, followed by ordinary one-way ANOVA plus Tukey's multiple comparison test for parametric data and Kruskal-Wallis test plus Dunn's multiple comparison test for non-parametric data. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$. Scale bar = 100 μm .

Finally, expression of the CSC-markers Nestin and SOX2 was assessed in all tumors. In Panc1 Holoclone tumors Nestin was strongly expressed (Frequency score 2-3 and Intensity score 4), significantly differing from Panc1 Paraclone tumors which showed no or only weak Nestin expression (Frequency- and Intensity score 1, Figure 20 A), which well reflected the different expression pattern identified *in vitro*. In line with the *in vitro* findings, both Panc89 Holoclone and Paraclone tumors showed no or only very faint Nestin expression (Figure 20 A).

In contrast to the *in vitro* findings, SOX2 expression could be detected in Panc1 Holoclone tumors (Frequency and Intensity score of 2/3) and expression levels were higher compared to Panc1 Paraclone tumors (Frequency and Intensity Score of 1/2, Figure 20 B). The expression of SOX2 in Panc89 Holoclone tumors was also significantly higher (Frequency and Intensity Score of 2/2) compared to Panc89 Paraclone tumors (Frequency and Intensity Score of 1/1) (Figure 20 B).



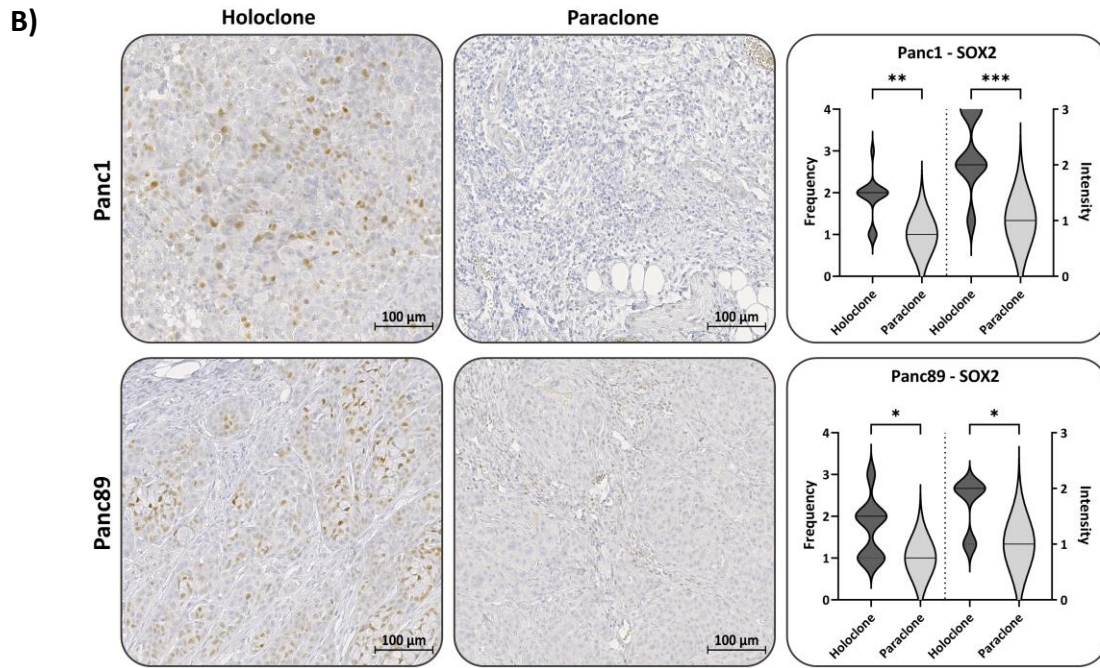


Figure 20 Immunohistochemical analysis of CSC-markers in tumor lesions of mice inoculated with Panc1 or Panc89 Holo- or Paraclone cells. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line) and extracted tissues and tumor lesions were stained for the CSC-markers A) Nestin and B) SOX2. Representative pictures of stained tissues of Panc1 and Panc89 Holo- or Paraclone tumors are presented in the left and middle picture panels, the right panel shows the statistical analysis of Frequency and Intensity score of positively stained cells. The upper row shows the data of analyzed Panc1 Holo- and Paraclone lesions, the lower panel shows the data of Panc89 Holo- and Paraclone lesions. Statistical analysis was performed by Shapiro-Wilk normality test, followed by ordinary one-way ANOVA plus Tukey's multiple comparison test for parametric data and Kruskal-Wallis test plus Dunn's multiple comparison test for nonparametric data. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$. Scale bar = 100 μm .

As mentioned above, 4/17 Panc89 Holoclone tumors showed areas of L1CAM expression. When correlating L1CAM and SOX2 expression in all tumors of this group, no significant correlation could be noted. However, analyzing Panc89 Holoclone tumors expressing L1CAM (Frequency score of 2-3) regarding SOX2, L1CAM expression in these tumors was significantly associated with low/absent SOX2 expression (Frequency score of 1) (Figure 21), being indicative of a phenotypic switch towards Paraclone cells. By examining the tissues of origin of these tumors it could be found that one tumor originated directly from the pancreas. The other examined tumors could not be clearly assigned to one organ, but were all surrounded by fat tissue, indicating localization of the tumors in the peritoneum.

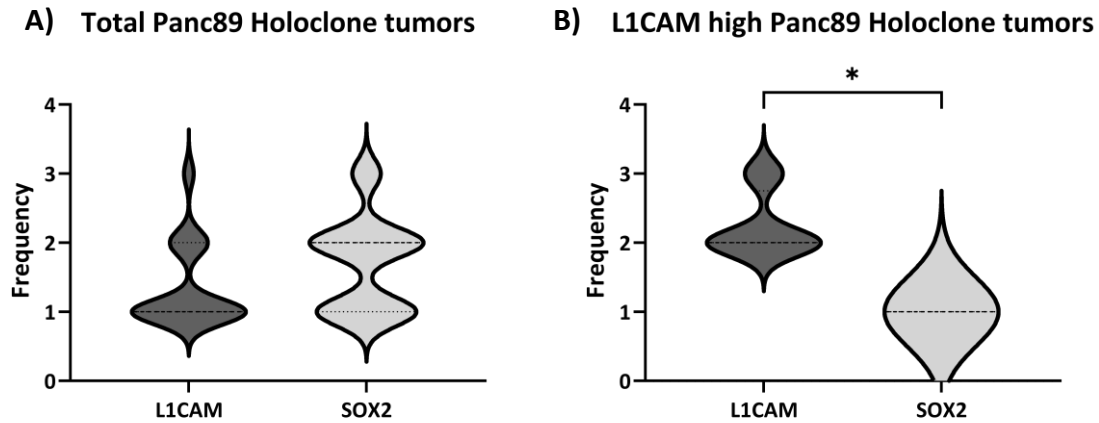


Figure 21 Immunohistochemical analysis of Panc89 Holoclone tumors that underwent a plasticity related switch *in vivo*. Mice were inoculated intrasplenically with 1×10^4 Panc89 Holoclone cells and L1CAM as well as SOX2 positive tumor lesions were analyzed. Comparative analysis of L1CAM and SOX2 expression in A) all Panc89 Holoclone tumors and B) of L1CAM expressing tumors are shown. Tumors formed by Panc89 Holoclone cells revealed a significant plasticity switch in 4/17 tumors, by downregulation of the CSC-marker SOX2 together with an increased expression of the mesenchymal marker L1CAM. Data were tested for normal distribution and afterwards Mann-Whitney test for non-parametric data was performed. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$.

In summary, IHC analysis widely confirmed the gene expression profile of the cells identified *in vitro*, approving mesenchymal Panc1 Holoclone cells with a Nestin-dominated CSC phenotype, while mesenchymal Panc1 Paraclone cells showing no stemness characteristics. Contrary, Panc89 Holoclone cells exhibited mostly an epithelial SOX2-dominated CSC phenotype, whereas Panc89 Paraclone cells exhibited a partial/hybrid EMT phenotype with less CSC-marker expression. However, certain variations from the expression patterns identified *in vitro* could be noted in Panc89 Holo- and Paraclone tumors (e.g., L1CAM, SOX2, Vimentin, ZEB1 and ZEB2) but also in Panc1 Holoclone cells (SOX2) underscoring the plasticity of the cells which seemed to be influenced by the tumor microenvironment.

Overall, these data indicate that the different CSC/EMT phenotypes of PDAC cell populations might yield different metastatic manifestations being associated with different survival times.

4.4 Different sequential treatments with cytostatic drugs of parental Panc1 and Panc89, Holo- and Paraclone cells

PDAC is a highly chemoresistant disease with a lack of effective therapeutic options. Besides the often advanced disease stage at time point of diagnosis ⁵⁻⁷, high tumor cell heterogeneity and plasticity achieved by the CSC-EMT-axis even aggravates therapy response and overall survival of PDAC patients ¹¹⁸⁻¹²¹. Therefore, studies on identifying appropriate subgroups of pancreatic cancers or subtypes of PDAC cells and their particularities in treatment response could offer novel approaches to improve therapy and outcomes of PDAC patients. In the last years, combination therapies with drugs like the combination regimen FOLFIRINOX or combination of nab-Paclitaxel and Gemcitabine gained more clinical relevance in treatment of PDAC patients with a good status of health ^{188,190,191,228,282-285}. Nevertheless, PDAC patients with an overall moderate or poor health status may be unable to tolerate the high toxicity of such combinational therapies. For this purpose, investigating treatment strategies with different drugs applied sequentially could reduce the risk of toxicity and improve quality of life. Furthermore, studies conducted with the use of chemotherapy holidays, aiming at avoiding the development of therapy resistances, have shown longer drug holidays in solid tumor treatment to provide a greater chance of response to re-treatments ^{221-224,286,287}, leading to consideration of sequential therapy options with or without drug holiday.

Thus, the following analysis aimed at a comparison of different combinations of the chemotherapeutic drugs Gemcitabine (G), Oxaliplatin (Ox) and 5-Fluorouracil (5-FU) with or without drug holiday, and how these affect cells with different CSC/EMT phenotypes. Therefore, parental Panc1 and Panc89, as well as Holo- and Paraclone cells were treated with each drug in monotherapy or in a sequential setting comprising two- or three of the cytostatic drugs, and the presence or absence of drug holidays. Furthermore, two time points (72 h, 120 h) for cell response analysis were determined.

4.4.1 Determination of the half maximal inhibitory concentration of Gemcitabine, Oxaliplatin and 5-Fluorouracil for parental Panc1 and Panc89, Holo- and Paraclone cells via Dose-Response-Analysis

The half maximal inhibitory concentration (IC₅₀) is a quantitative parameter indicating how much of a specific agent is required to inhibit e.g. biological processes by 50%²⁸⁸. To determine IC₅₀ values of the cytostatic drugs G, Ox, and 5-FU, a Dose-Response-Analysis (DRA) was performed.

Parental Panc1 and Panc89, Holo- and Paraclone cells were seeded and after 24 h the cells were treated with different concentrations of G, Ox and 5-FU (Figure 22). Cell confluence was determined before the start of treatment (=T₀) and the cells were stimulated for 72 h with each drug. The cell confluence was measured and nuclei staining with Hoechst33342 (Figure 22) as well as dead cell staining with Propidium iodide (PI) was performed to analyze the change in cell confluence, number of cell nuclei and number of dead cells. IC₅₀ values for G, Ox and 5-FU were determined using percentage of Hoechst33342 stained cells normalized to the NaCl control (CTRL). As the PDAC cell lines changed their morphology during the treatment, the cell confluence was finally not considered for determination of IC₅₀ concentration. Moreover, as apoptotic cells detached from cell culture plates and thus, despite positive PI staining, could no longer be detected, analysis of dead cells was also not considered as reliable parameter. Nevertheless, cell confluence as well as cell death analysis were statistically analyzed and are presented in supplementary data (Supplementary figure 3, 5, 6).

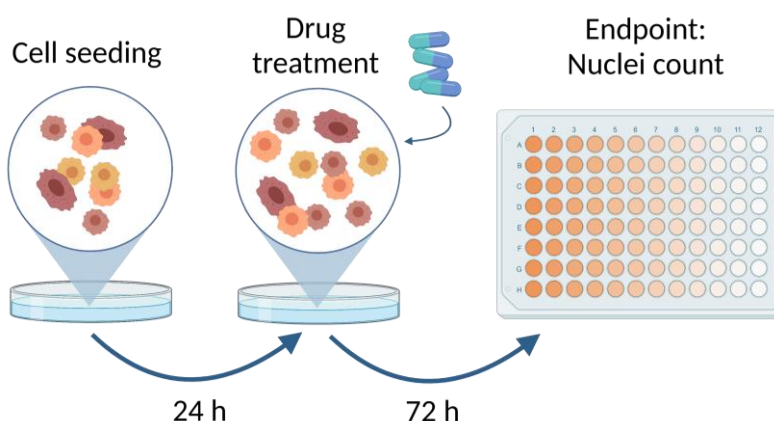


Figure 22 Schematic representation of the experimental procedure for determining the half maximal inhibitory concentrations of the cytostatic drugs Gemcitabine, Oxaliplatin and 5-Fluorouracil. 0.5×10^4 of parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates. After 24 h, the cells were treated with nine different concentrations of either cytostatic drug or the carrier solution (NaCl) as control (CTRL) for 72 h. Afterwards, the amount of cell nuclei was analyzed by Hoechst33342 staining. The nuclei count analysis was used to

determine the IC50 values by [Inhibitor] vs. response - Nonlin-fit in GraphPad Prism. Created with BioRender.com.

The analysis revealed an IC50 value for G of 0.0038 μ M for both parental Panc1 and Panc89, as well as their Holo- and Paraclone cells.

Based on previous studies showing a therapy resistant phenotype of Panc1 cells ^{65,78}, a high concentration of 38 μ M G was chosen as maximum dose for the IC50 determination of Panc1 parental, Holo- and Paraclone cells. It could be shown that in a dose range of 38 – 0,038 μ M G none of the Panc1 cell lines were affected (Figure 23 A).

For parental Panc89, Holo and Paraclone cells lower concentrations for the determination of the IC50 were chosen with a maximum dose of 0.6 μ M G. All Panc89 cell lines were already affected at lower G concentrations compared to Panc1 cell lines, but in conclusion the IC50 value for all cell lines turned out to be the same (0.0038 μ M; Figure 23 B).

IC50 for Ox of parental Panc1 and Panc1 Paraclone cells was 0.5 μ M and for Panc1 Holoclone cells 0.25 μ M (Figure 23 C). For all Panc89 cell lines the same IC50 of 0.8 μ M Ox could be determined (Figure 23 D).

IC50 determination for 5-FU resulted in different values for all analyzed cell lines. Parental Panc1 cells showed an IC50 value of 0.8 μ M, Panc1 Holoclone cells of 0.08 μ M and Panc1 Paraclone of 0.04 μ M 5-FU (Figure 23 E). 5-FU IC50 of parental Panc89 cells was 0.2 μ M, for Panc89 Holoclone cells 1.5 μ M and for Panc89 Paraclone cells 0.08 μ M (Figure 23 F).

Treatment with G obviously did not show differences in the affection of epithelial and mesenchymal cells as well CSCs and non-CSCs. Treatment with Ox led to a worse drug response in epithelial cells compared to mesenchymal cells, and within the mesenchymal Panc1 cell lines it could be determined that Holoclones showed the strongest affection. *Vice versa*, comparing parental epithelial and mesenchymal cells under treatment with 5-FU it was found that mesenchymal cells showed poorer responses compared to epithelial cells. Furthermore, both Panc1 and Panc89 Paraclone cells showed a better response to 5-FU compared to their related Holoclone cells. These data already indicated that certain differences in treatment response occur between epithelial and mesenchymal PDAC cells (e.g., Ox and 5-FU) and within isolated CSC and non-CSC variants (e.g., 5-FU treatment of Panc89 Holo- and Paraclones or Ox treatment of Panc1 Holo- and Paraclones).

Examined IC50 values of G, 5-FU and Ox were used for all following schedules with monotreatment or sequential therapy.

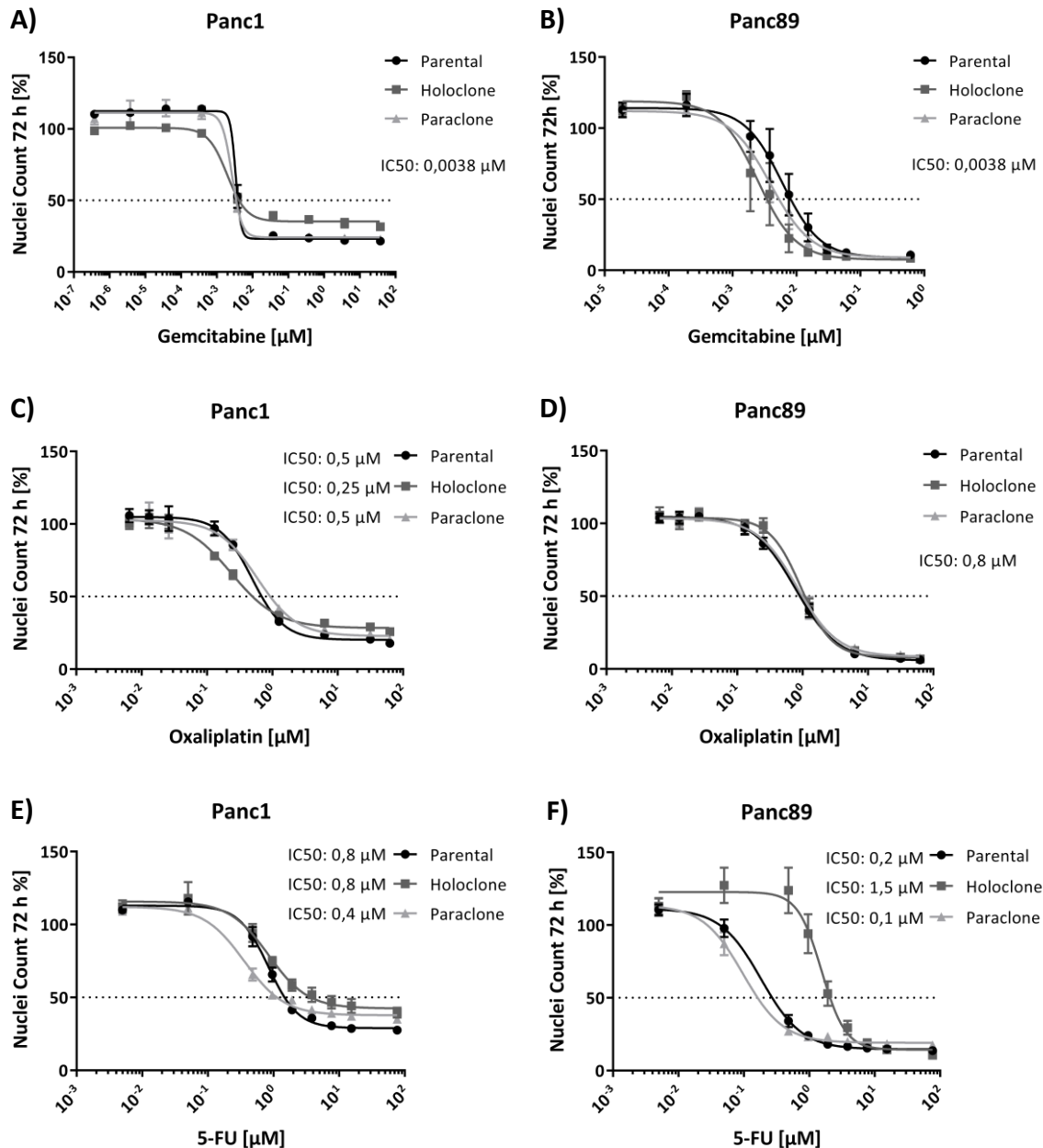


Figure 23 Determination of the half maximal inhibitory concentration for parental Panc1 and Panc89, Holo- and Paraclone cells for the cytostatic drugs Gemcitabine, Oxaliplatin and 5-Fluorouracil. For maximal inhibitory concentration (IC₅₀) determination 0.5×10^4 of each parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates. After 24 h, the cells were treated with nine different concentrations of cytostatic drugs or were left treated with NaCl as CTRL. Afterwards, the cell confluence was monitored every 24 h. Finally, after 72 h of stimulation, an endpoint analysis was performed by measuring the amount of cell nuclei by Hoechst33342 staining. The nuclei count analysis was used to determine the IC₅₀ values by [Inhibitor] vs. response Nonlin-fit in GraphPad Prism. The graphs present the percentage of nuclei count after 72 h treatment normalized to NaCl control (Y-axis) and used concentration of respective cytostatic agents in μM (X-axis). IC₅₀ determination for A, B) Gemcitabine, C, D) Oxaliplatin and E, F) 5-FU for A, C, E) parental Panc1, Holo- and Paraclone cells as well as B, D, F) parental Panc89, Holo- and Paraclone cells. Independent experiments n = 3 - 4.

4.4.2 Short-term sequential therapies (72 h) of parental Panc1 and Panc89, Holo- and Paraclone cells with the cytostatic drugs Gemcitabine, Oxaliplatin and 5-Fluorouracil

4.4.2.1 Short-term sequential therapy of parental Panc1 and Panc89, Holo- and Paraclone cells with two cytostatic drugs without drug holiday

As a first treatment strategy a short-term sequential therapy for 72 h comprising treatment with two drugs without drug holiday was performed. Since no effects from the NaCl CTRL was detected on the PDAC cell variants during the IC₅₀ determination, untreated cells were used as CTRL from here on and monotherapy using only either cytostatic drug following the same therapy scheme was also carried out. Parental Panc1 and Panc89, Holo- and Paraclone cells were seeded and treatment started by applying the first cytostatic drug for 24 h. Next, treatment with the second drug was performed for 48 h. After a total therapy time of 72 h, the nuclei count endpoint analysis with Hoechst33342 was performed (Figure 24), as well as determination of cell confluence and cell death (PI) analysis (Supplementary figure 7).

Short-term sequential therapy (72 h, 2 drugs without drug holiday)

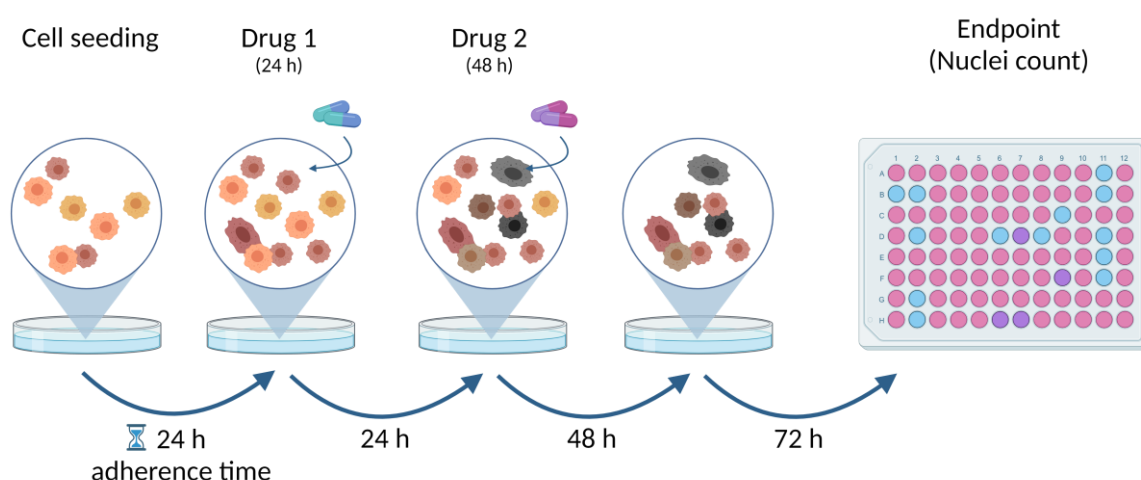


Figure 24 Illustration of short-term sequential therapy for 72 h without drug holiday. 0.5×10^4 Panc1 and Panc89 parental, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated with the first cytostatic drug (G, Ox or 5-FU) for 24 h or left untreated as CTRL. After 24 h, the drugs were removed and after a washing step the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 48 h. Cell confluence was monitored every 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as Propidium iodide (for detection of dead cells) and Hoechst33342 (for detection of cell nuclei) staining. The statistical evaluation was performed with the nuclei count data *via* Hoechst33342 staining. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil). Created with BioRender.com.

As expected by determined IC₅₀ values, monotherapy with G - G led to about 50% decrease of nuclei counts in all Panc1 cell lines (Figure 25 A), albeit the parental cells responded weakly better than the derived Holo- and Paraclone cells. Monotherapy with Ox - Ox resulted in approximately 50% decrease in nuclei count in parental Panc1 cells, whereas in Panc1 Holo- and Paraclone cells a decrease of 20-30% was observed (Figure 25 A). Monotherapy with 5-FU - 5-FU only slightly decreased number of nuclei by 10-25% in parental Panc1, Holo- and Paraclone cells (Figure 25 A). G - G monotherapy led to a strong, significant decrease of nuclei in all Panc89 cell lines of about 75-90%, whereas monotherapy with 5-FU - 5-FU only decreased the nuclei count by about 25% (Figure 25 B). Monotherapy with Ox - Ox led to a similar affection of parental Panc89, Holo and Paraclone cells with a decreased nuclei count of about 60% for all cell lines (Figure 25 B).

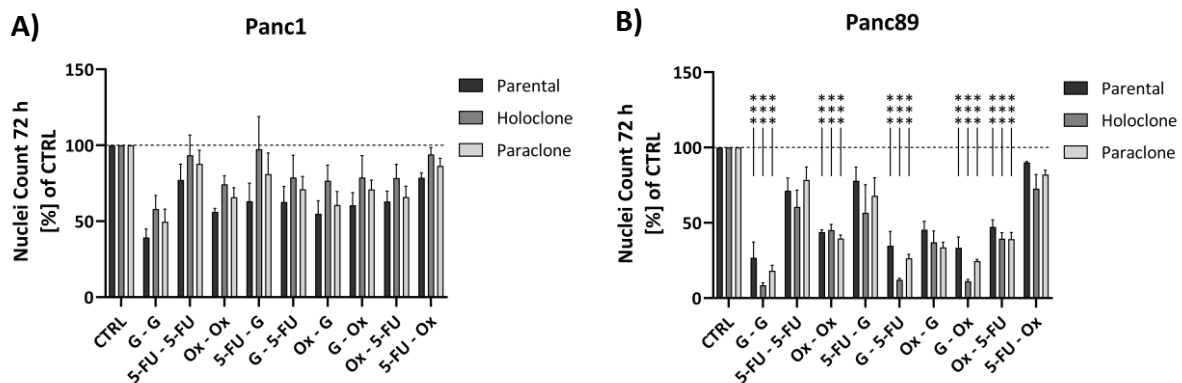


Figure 25 Short-term sequential therapy for 72 h without drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs were replaced with the second cytostatic drug (G, Ox or 5-FU) for 48 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by Hoechst33342 (for detection of cell nuclei) staining for A) parental Panc1, Holo- and Paraclone cells as well as B) parental Panc89, Holo- and Paraclone cells. The graphs show the percentage of analyzed nuclei count normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, independent experiments $n = 3$. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil)

In parental Panc1, Holo- and Paraclone cells, none of the sequential treatments resulted in a greater decrease in nuclei counts compared to G - G monotherapy. Although no significant results were observed for short-term sequential therapy without drug holiday, Panc1 Holo-clones appeared to be less affected by sequential treatment compared to parental Panc1 and Panc1 Paraclone cells (Figure 25 A).

Short-term sequential therapy without drug holiday of Panc89 cell lines led to a variety of significant effects of the different treatments, but treatment with 5-FU - G or 5-FU - Ox could not lead to significant nuclei count decreases. Only Panc89 Holo-clones, even if not significant, showed approximately 50% nuclei count decrease after sequential treatment with 5-FU - G. Significant nuclei count decrease could be determined after sequential therapy with G - 5 FU or G - Ox in all Panc89 cell variants. Nevertheless, the reduction in nuclei count after G - 5-FU or G - Ox treatment was not stronger compared to G - G monotherapy. However, the results showed that any treatment with G applied as first drug, regardless of mono- or sequential therapies, had most significant effects on the nuclei decrease in all Panc89 cell variants (Figure 25 B).

In conclusion, comparing Panc1 and Panc89 cells, it could be found that Panc1 Holoclone cells are less affected by sequential therapy without drug holiday compared to related parental and Paraclone cells, while Panc89 Holo-clones are the most affected cells within Panc89 cell populations. Overall, treatment effects were stronger in Panc89 cell variants compared to Panc1 cell variants.

4.4.2.2 Short-term sequential therapy of parental Panc1 and Panc89, Holo- and Paraclone cells with two cytostatic drugs with drug holiday

As a second treatment strategy a short-term sequential therapy for 72 h comprising treatment with two drugs and a drug holiday was applied. Untreated cells were used as CTRL and monotherapy using only either cytostatic drug following the same therapy scheme was also carried out. After seeding parental Panc1 and Panc89, Holo- and Paraclone cells, treatment was started by applying the first cytostatic drug for 24 h. After removal of the drugs, a drug holiday was applied. After 24 h, cells were treated with the second cytostatic drug for another 24 h. After a total therapy time of 72 h, the nuclei count endpoint analysis with Hoechst33342 was performed (Figure 26), as well as determination of cell confluence and cell death (PI) analysis (Supplementary figure 6).

Short-term sequential therapy (72 h, 2 drugs with drug holiday)

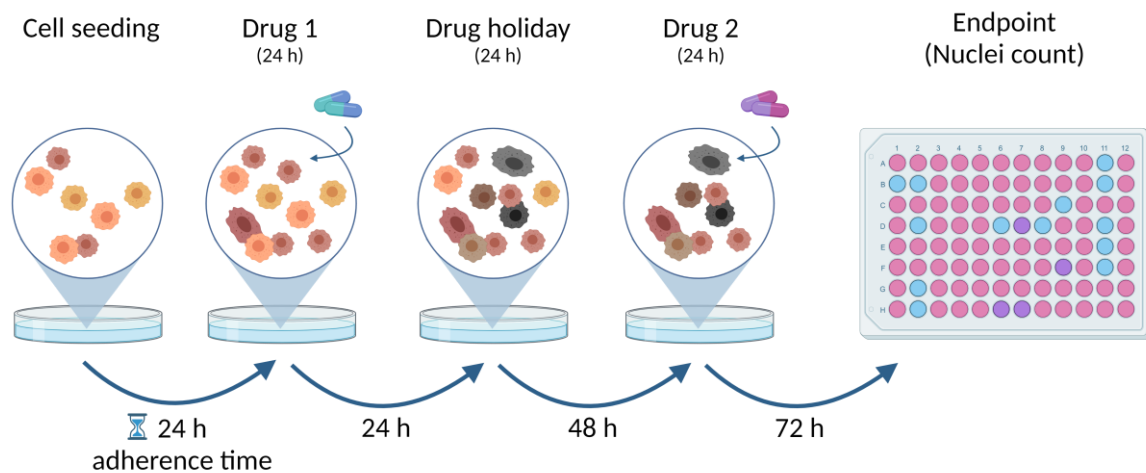


Figure 26 Illustration of short-term sequential therapy for 72 h with drug holiday. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated with the first cytostatic drug (G, Ox or 5-FU) for 24 h or left untreated as CTRL. After 24 h, the drugs were removed and after a washing step replaced by fresh cell culture medium for a 24 h drug holiday. After drug holiday, medium was removed and the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 24 h. Cell confluence was monitored every 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as cell staining with Propidium iodide (for detection of dead cells) and Hoechst33342 (for detection of cell nuclei). The statistical analysis was performed with the nuclei count data *via* Hoechst33342 staining. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil). Created with BioRender.com.

First, although for all cytostatic drugs IC₅₀ concentrations were applied, the decrease in nuclear cell number was not 50% after monotherapies, except for Panc89 Holo- and Paraclone cells treated with G - G monotherapy. After treatment with the other monotherapies, Ox - Ox or 5-FU - 5-FU, applied to parental Panc1, Holo- and Paraclone cells as well as parental Panc89 cells, the decrease in nuclei count was less than 25% (Figure 27). This discrepancy to the DRA might be due to drug holiday reducing the time of drug application to the cells. Furthermore, application of a single drug with drug holiday was less effective than treatment without drug holiday.

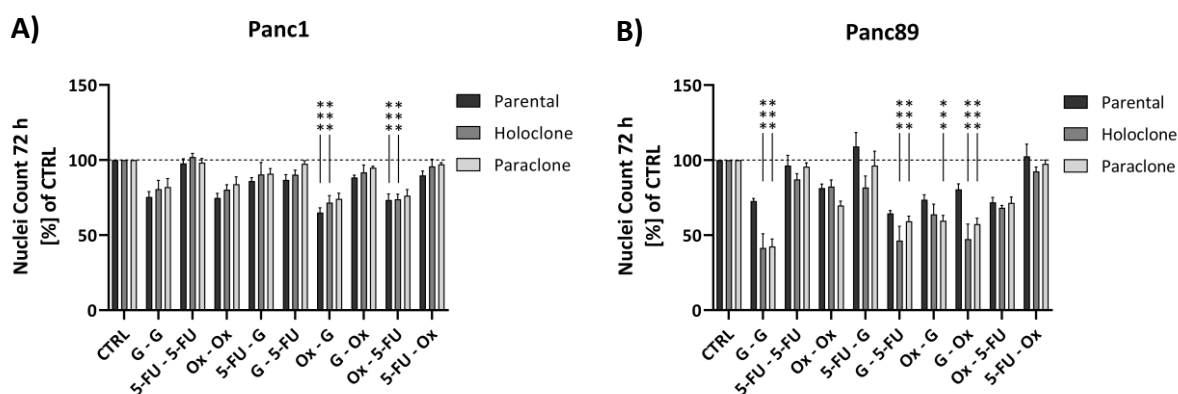


Figure 27 Short-term sequential therapy for 72 h with drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs were replaced with fresh cell culture medium for 24 h drug holiday. After drug holiday, the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by Hoechst33342 (for detection of cell nuclei) staining for A) parental Panc1, Holo- and Paraclone cells as well as B) parental Panc89, Holo- and Paraclone cells. The graphs show the percentage of analyzed nuclei count normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, independent experiments $n = 4$. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil)

Parental Panc1, Holo and Paraclone cells showed treatment responses, even though not significant, by decreased nuclei count to monotherapy with G - G or Ox - Ox, but no response to 5-FU - 5-FU treatment. Therapy with 5-FU basically showed no effect on Panc1 cell populations, neither after mono- nor sequential therapy, with one exception for Ox - 5-FU, leading to a decrease in nuclei count of about 25% in parental Panc1 and Panc1 Holoclone cells. Furthermore, treatment with Ox - Ox or G - G monotherapy led to a reduction of cell nuclei, but compared to sequential therapy it could be found that the combination of Ox with G or with 5-FU, applied as second drug, led to a significant decrease of cell nuclei of about 25% in parental Panc1 as well as Panc1 Holoclone cells (Figure 27 A).

In summary, Panc1 cell lines showed a better response to sequential treatment when Ox was applied as first drug, significantly for parental Panc1 and Holoclone cells, but overall, no major differences could be detected comparing treatment response of parental Panc1 and Holoclone cells to response of Panc1 Paraclone cells. The strongest affection could be detected in the heterogeneous parental Panc1 cells after treatment with Ox - G (Figure 27 A). All in all, the three Panc1 cell variants responded relatively similar to applied treatment strategies, with a slightly poorer response of Paraclone cells.

By contrast, Panc89 cells showed different responses to short-term sequential treatment with drug holiday (Figure 27 B). Comparing the monotherapies, the strongest effect could be found after G - G, with a significant decrease of cell nuclei of at least 50-60% in Panc89 Holo- and Paraclone cells. Parental Panc89 cells were less affected with a not significant nuclei count decrease of about 25% by G - G monotherapy. Monotherapies with 5-FU - 5-FU or Ox - Ox led to even poorer effects, as the maximum decrease of cell nuclei by approximately 25% could be induced by Ox - Ox in Panc89 Paraclone cells. Sequential therapies with 5-FU - G or 5-FU - Ox did not affect Panc89 cell populations, being in line with the data of short-term sequential therapy without drug holiday. Sequential treatment with Ox - 5-FU led to a decrease in nuclei count for all Panc89 cell lines, but was comparatively weak. The strongest effects could be detected after sequential therapies with G - 5-FU or G - Ox in Panc89 Holo- and Paraclone cells with a significant nuclei reduction of about 50%. Furthermore, Panc89 Paraclones showed a significant decrease in nuclei count after the sequential treatment with Ox - G, underlining the better effect of Ox in cells with mesenchymal properties. Even though no significant differences could be examined for parental Panc89 cells, the data showed sequential therapy with G - 5-FU to be the most promising treatment leading to a decreased nuclei count of about 30%. Overall, Panc89 cell populations showed the strongest affection by monotherapy with G - G or any sequential therapy when G was applied as first drug, concluding G - 5-FU to be the most effectful sequential therapy (Figure 27 B). Furthermore, Panc89 Holo- and Paraclone cells showed overall higher response rates to tested therapies compared to the heterogeneous parental cells.

4.4.2.3 Short-term sequential therapy of parental Panc1 and Panc89, Holo- and Paraclone cells with three cytostatic drugs without drug holiday

The last experimental setting of short-term sequential therapy experiments for 72 h was performed without a drug holiday and with three drugs. Untreated cells were used as CTRL and treatment with monotherapy was also carried out. Parental Panc1 and Panc89, Holo- and Paraclone cells were seeded and therapy started by treatment of the cells with the first cytostatic drug for 24 h, followed by treatment with the second drug for 24 h and finally, ended with 24 h treatment with the third drug. Between treatments the cells were washed with fresh cell culture medium to remove remnants of the previous drugs. After a total therapy time of 72 h,

the nuclei count endpoint analysis with Hoechst33342 was performed (Figure 28) as well as determination of cell confluence and cell death (PI) analysis (Supplementary figure 8).

Short-term sequential therapy (72 h, 3 drugs without drug holiday)

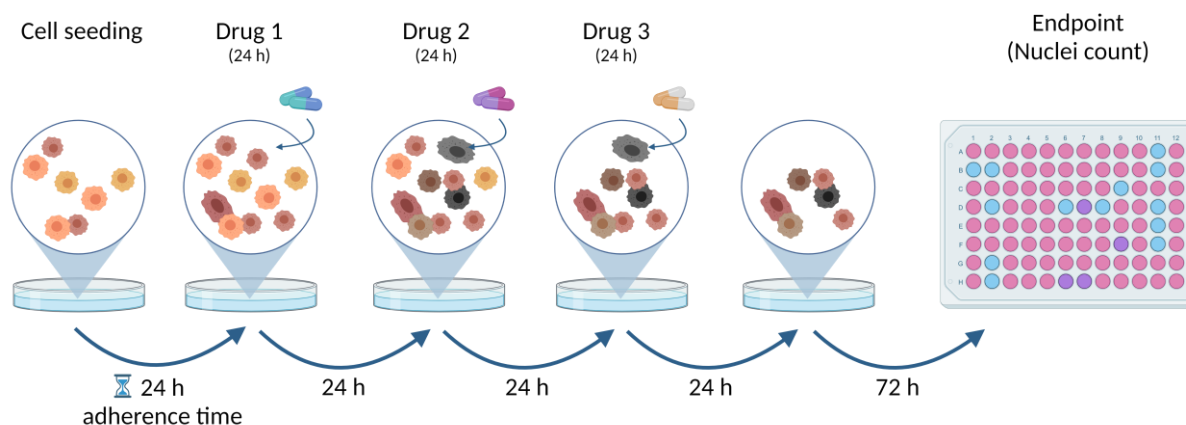


Figure 28 Illustration of short-term sequential therapy for 72 h without drug holiday and use of three cytostatic drugs. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated with the first cytostatic drug (G, Ox or 5-FU) for 24 h or left untreated as CTRL. After 24 h, the drugs were removed and after a washing step the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for another 24 h. Afterwards, the cells were washed again and finally treated with the third cytostatic drug (G, Ox or 5-FU) for additional 24 h. Cell confluence was monitored every 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by again measuring the cell confluence as well as Propidium iodide (for detection of dead cells) and Hoechst33342 (for detection of cell nuclei) staining. The statistical evaluation was performed with the nuclei count data *via* Hoechst33342 staining. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil). Created with BioRender.com.

Monotherapy with G led to a decreased nuclei number of almost exactly 50% in parental Panc1 and Panc1 Paraclone cells, while Panc1 Holoclones were slightly less affected presented by nuclei count decrease of about 40% (Figure 29 A). Monotherapy with Ox also led to a reduction of nuclei counts of about 25-45% in all Panc1 cell lines, with a slightly worse response in Panc1 Holoclone cells (Figure 29 A). Basically, results for the monotreatment with G and Ox were consistent with observations from the IC50 determination. This was again not the case for 5-FU, since there was no effect on all Panc1 cell lines (Figure 29 A).

Monotherapy with G in Panc89 cell lines led to a significant decrease in nuclei count of 80% in parental and Paraclone cells and 90% in Holoclone cells (Figure 29 B). Ox monotreatment led to a decrease of cell nuclei in parental Panc89, Holo and Paraclone cells of almost 50%, corresponding to the determination of IC50 concentration (Figure 29 B). Monotherapy with 5-FU decreased the nuclei count by about 20% in all Panc89 cell lines (Figure 29 B)

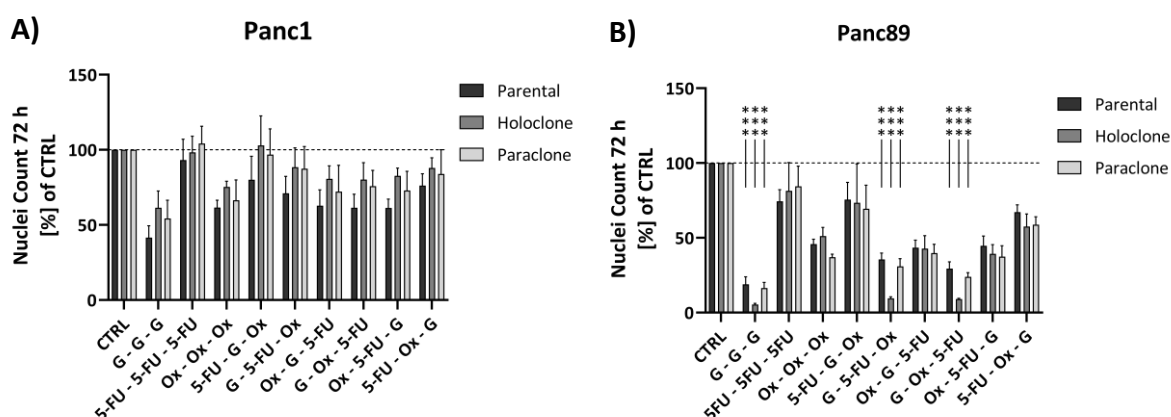


Figure 29 Short-term sequential therapy for 72 h with 3 cytostatic drugs without drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs were replaced with the second cytostatic drug (G, Ox or 5-FU) for another 24 h. Finally, the cells were treated with the third cytostatic drug (G, Ox or 5-FU) for additional 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by Hoechst33342 (for detection of cell nuclei) staining for A) parental Panc1, Holo- and Paraclone cells as well as B) parental Panc89, Holo- and Paraclone cells. The graphs show the percentage of analyzed nuclei count normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, independent experiments $n = 3$. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil)

In parental Panc1, Holo- and Paraclone cells none of the sequential treatments led to stronger decreases of nuclei counts when compared to G monotherapy, as already determined in short-term therapy with two drugs and without drug holiday (4.4.2.1). Although no significant effects were observed, Panc1 Holoclone cells generally appeared to be less affected by treatment compared to parental Panc1 and Paraclone cells (Figure 29 A), which could also be observed after short-term sequential therapy with two drugs and abundance of drug holiday (4.4.2.1).

Short-term therapy with three drugs in the absence of drug holiday led to significant responses in Panc89 cell lines, however, being similar to those observed after short-term therapy with two drugs and without drug holiday (4.4.2.1). In each treatment strategy, pre-treatment with G, independent of mono- or sequential treatment, led to the strongest decrease in nuclei count in Panc89 cell variants (Figure 29 B). Of note, Panc89 Holoclone cells showed the strongest response when G was used as first drug in mono- and sequential treatment (Figure 29 B). Thus, these data indicate that epithelial cells in general but particularly cells with an epithelial

CSC-phenotype (Panc89 Holoclones) might be mostly sensitive to G based therapies. Furthermore, it was found that a drug holiday-based therapy strategy reduced the sensitivity to treatment in Panc1 and Panc89 cell variants (4.4.2.2), except for Panc1 Holoclone cells.

4.4.3 Long-term sequential therapies (120 h) of parental Panc1 and Panc89, Holo- and Paraclone cells with the cytostatic drugs Gemcitabine, Oxaliplatin and 5-Fluorouracil

4.4.3.1 Long-term sequential therapy of parental Panc1 and Panc89, Holo and Paraclone cells with three cytostatic drugs and without drug holiday

In the following treatments duration was expanded from 72 h to 120 h with or without drug holiday. Like in previous experiments, untreated cells were used as CTRL and monotherapy using only one cytostatic drug but following the same therapy scheme was also carried out. First, long-term sequential therapy applying all three drugs (G, 5-FU, Ox) without drug holiday was tested, started by treatment of the cells with the first cytostatic drug for 24 h. After a washing step, treatment with drug two was carried out for 48 h. Afterwards, cells were washed and treated with the third drug for another 48 h. After a total therapy time of 120 h, the nuclei count endpoint analysis with Hoechst33342 was performed (Figure 30) as well as determination of cell confluence and cell death (PI) analysis (Supplementary figure 10).

Long-term sequential therapy (120 h, 3 drugs without drug holiday)

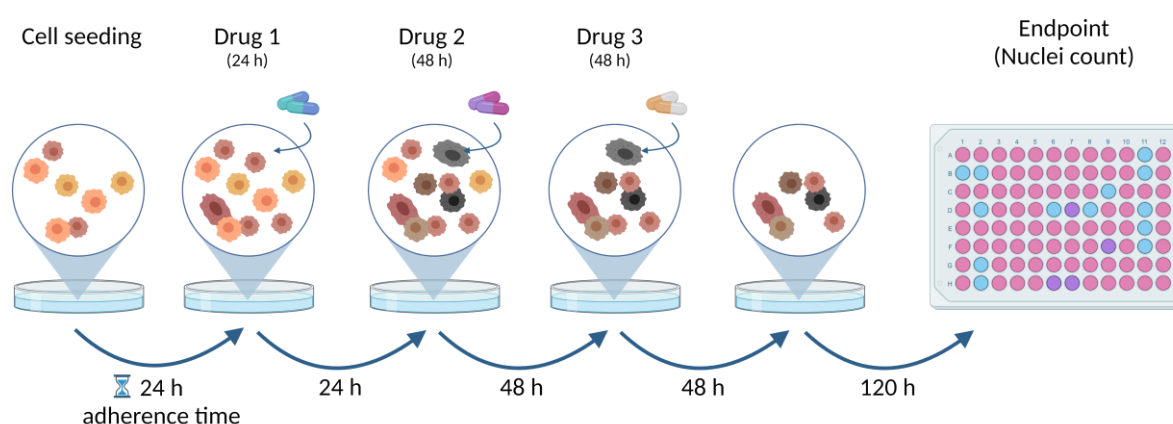


Figure 30 Illustration of long-term sequential therapy for 120 h with three cytostatic drugs without drug holiday. 0.25×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated with the first cytostatic drug (G, Ox or 5-FU) for 24 h or left untreated as CTRL. After 24 h, the drugs were removed and after a washing step the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for 48 h. Afterwards, the cells were washed and finally treated with the third cytostatic drug (G, Ox or 5-FU) for additional 48 h. Cell confluence was monitored every 24 h. After a total therapy time of 120 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as Propidium iodide (for detection of dead cells) and Hoechst33342 (for detection of cell nuclei) staining. The statistical

- Results -

analysis was performed with the nuclei count data *via* Hoechst33342 staining. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil). Created with BioRender.com.

In Panc1 cell lines the monotreatment with 5-FU did not show any effects. Monotherapy with G or Ox led to decreased in nuclei count of minimum 50% (Figure 31 A). Interestingly, in this long-term sequential therapy strategy Panc1 Holoclone cells were more affected by monotherapy compared to Paraclone cells, differing from previous data of short-term sequential therapies. For sequential treatments of parental Panc1, Holo- and Paraclone cells it was found that applying 5-FU - G - Ox or G - 5-FU - Ox showed no effects on the cells (Figure 31 A). Every other drug combination led to similar effects on reduction of cell nuclei, with a slightly better effect of sequential therapy with Ox - G - 5-FU (Figure 31 A). It could also be determined that Panc1 Holoclone cells showed stronger treatment responses after sequential therapies applied for 120 h compared to short-term therapies.

Monotherapy in Panc89 cell lines led to a decrease in nuclei count of about 25% after 5-FU treatment, with the strongest effects on Panc89 Holoclones. Monotherapy with Ox decreased the nuclear number of about 40-50% in Panc89 cell lines, with the strongest decrease in nuclei count for Panc89 Paraclones. Monotherapy with G led to a decrease of nuclei count in parental Panc89 cells of about 60%, while Panc89 Holo- and Paraclone cells were strongly affected shown by nuclei count decrease of 80-90% (Figure 31 B). Overall, parental Panc89 cells were less affected by monotherapy compared to Panc89 Holo- and Paraclone cells. For sequential treatments it could be found that application of G - Ox - 5-FU led to the strongest decrease of nuclei counts in all Panc89 cell lines, with a slightly stronger response in Panc89 Holo- and Paraclone cells. Treatment with G - Ox - 5-FU showed nuclei count decrease in parental Panc89 cells which was comparable to the effects of G monotherapy, while Panc89 Holo- and Paraclone cells were less affected compared to G monotreatment (Figure 31 B). Furthermore, differences between the clonal populations of Panc89 cells could be found for the different long-term treatments without drug holiday. Panc89 Holoclone cells showed a stronger treatment response to G and Ox monotherapy and sequential treatments with G - 5-FU - Ox, Ox - 5-FU - G or 5-FU - Ox - G compared to Panc89 Paraclones. Parental cells overall showed weaker treatment response than Panc89 Holo- and Paraclone cells (Figure 31 B).

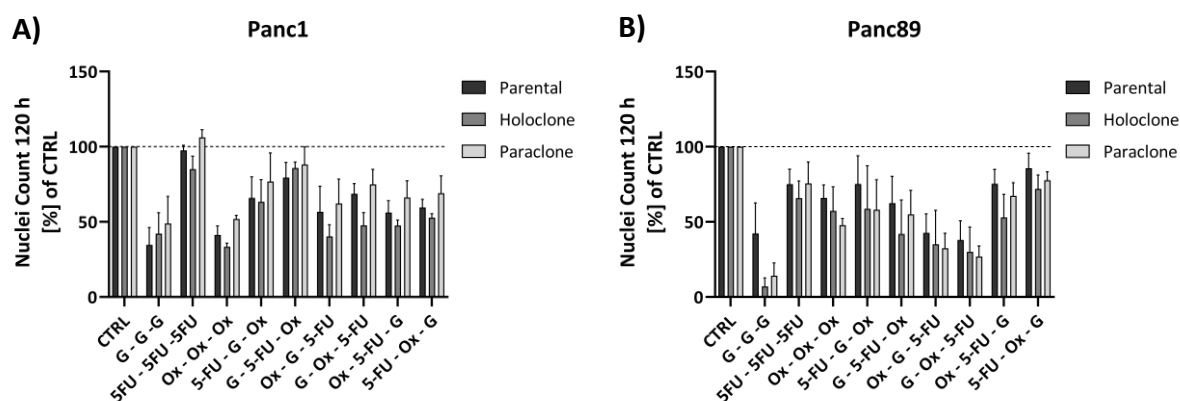


Figure 31 Long-term sequential therapy for 120 h using 3 cytostatic drugs without drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.25×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or were left untreated as CTRL. After 24 h, the drugs were replaced with the second cytostatic drug (G, Ox or 5-FU). After 48 h, the cells were treated with the third cytostatic drug (G, Ox or 5-FU) for additional 48 h. After a total therapy time of 120 h the experiment was terminated with an endpoint analysis by Hoechst33342 (for detection of cell nuclei) staining for A) parental Panc1, Holo- and Paraclone cells as well as B) parental Panc89, Holo- and Paraclone cells. The graphs show the percentage of analyzed nuclei count normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, independent experiments $n = 3$. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil)

4.4.3.2 Long-term sequential therapy of parental Panc1 and Panc89, Holo- and Paraclone cells with two cytostatic drugs and with drug holiday

Next, a long-term sequential treatment using two drugs and applying drug holiday was performed. For this purpose, parental Panc1 and Panc89, Holo- and Paraclone cells were seeded and after 24 h, the treatment started by applying the first cytostatic drug for 72 h. After a washing step, drug holiday with fresh cell culture medium was carried out for 24 h, followed by treatment with the second drug for further 24 h. After a total therapy time of 120 h, the nuclei count endpoint analysis with Hoechst33342 was performed (Figure 32) as well as determination of cell confluence and cell death (PI) analysis (Supplementary figure 9).

Long-term sequential therapy (120 h, 2 drugs with drug holiday)

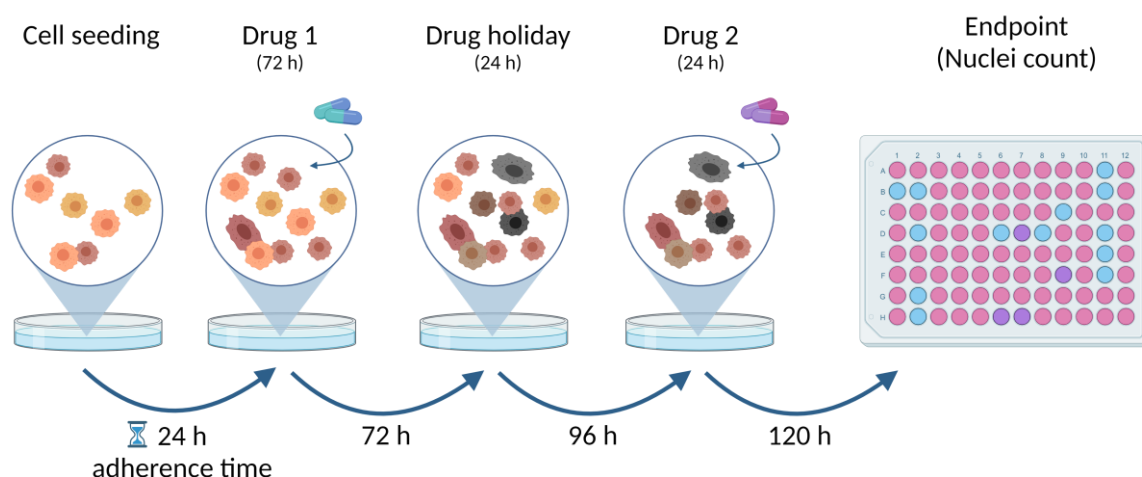


Figure 32 Illustration of long-term sequential therapy for 120 h with drug holiday. 0.25×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated with the first cytostatic drug (G, Ox or 5-FU) for 72 h or left untreated as CTRL. After 72 h, the drugs were removed and after a washing step fresh cell culture medium was added for 24 h drug holiday. After 24 h, medium was removed and the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 24 h. Cell confluence was monitored every 24 h. After a total therapy time of 120 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as Propidium iodide (for detection of dead cells) and Hoechst33342 (for detection of cell nuclei) staining. The statistical analysis was performed with the nuclei count data *via* Hoechst33342 staining. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil). Created with BioRender.com.

First of all, none of the monotherapies resulted in a 50% reduction in cell nuclear number in Panc1 and Panc89 cell variants, regardless of clone-type, although treatments with the first drug lasted 72 hours, the same duration used to determine IC50 values. Monotherapy with G and 5-FU in Panc1 cell lines led to a reduction of nuclei counts of about 20%, with a similar response of parental Panc1, Holo- and Paraclone cells. Monotreatment with Ox led to the strongest nuclei count decrease in parental Panc1 cells, with a decrease of about 40%, followed by Panc1 Holoclone cells with a decrease of 25% and with the weakest effects in Panc89 Paraclones with nuclei count decrease of about 20% (Figure 33 A). For the sequential therapies it was found that Ox - G and Ox - 5-FU induced the strongest effects in Panc1 cell lines, with the best response in parental Panc1 cells (Figure 33 A). These data are in line with previous sequential treatment data, showing strongest effects on Panc1 cell lines under first treatment with Ox.

Monotherapy with G in Panc89 cell lines reduced the nuclei count of parental and Holoclone cells by 25%, while Panc89 Paraclone cells showed stronger nuclei count reduction of 50%. Monotreatment with 5-FU led to a decrease in nuclei counts of about 15-20% in all Panc89

cell lines. Ox monotherapy decreased the nuclei count by 20-25% in parental Panc89 and Panc89 Paraclone cells, while in Panc89 Holoclones nuclei numbers were even increased compared to CTRL (Figure 33 B). Sequential therapies showed no effects on Panc89 cell variants when the treatment started with application of 5-FU as first drug. Sequential treatment with Ox - G showed the same effects as monotherapy with Ox, with an even increased nuclei count in Panc89 Holoclones compared to CTRL (Figure 33 B). Furthermore, sequential treatment with Ox - 5-FU again could not reduce the nuclei of Panc89 Holoclones, while parental Panc89 and Panc89 Paraclone nuclei counts were reduced by about 20-25%. Strongest effects could be found after G - 5-FU or G - Ox treatment, with a decreased nuclei count in parental Panc89 and Paraclone cells of about 25% and about 20% in Panc89 Holoclones (Figure 33 B). Overall, these data again indicate that applying drug holidays in mono- or sequential therapies leads to a reduction of treatment response in PDAC cells, except for treatment with 5-FU being overall less effective. This particularly applies for Panc89 Holoclone cells, where long-term sequential treatment with drug holiday led to an even increased nuclei number compared to CTRL for some of the treatment strategies.

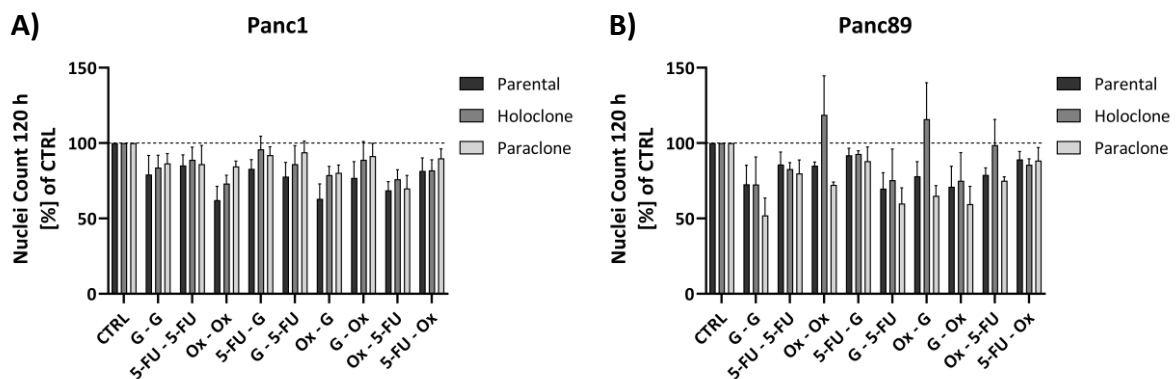


Figure 33 Long-term sequential therapy for 120 h with drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.25×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 72 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 72 h, the drugs were replaced with fresh cell culture medium for 24 h drug holiday. After drug holiday, the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 24 h. After a total therapy time of 120 h, the experiment was terminated with an endpoint analysis by Hoechst33342 (for detection of cell nuclei) staining for A) parental Panc1, Holo- and Paraclone cells as well as B) parental Panc89, Holo- and Paraclone cells. The graphs show the percentage of analyzed nuclei count normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple compari-

- Results -

sons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, independent experiments n = 5. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil)

In summary, it could be determined that drug holiday reduced the effects of mono- and sequential therapies in both Panc1 and Panc89 cell lines independent of short-term or long-term sequential treatment strategies. However, compared to parental Panc1 and Panc1 Paraclone cells, Panc1 Holoclone cells appeared to be more affected by sequential therapy with drug holiday, independent of short- or long-term treatment, than every other sequential therapy, to which Panc1 Holoclones showed poorer responses compared to parental Panc1 and Panc1 Paraclone cells. Overall, short-term therapy applying two drugs without drug holiday was found to be best strategy for Panc1 cell lines, with the strongest responses to G monotherapy. Comparing short-term and long-term sequential therapies for Panc1 cell lines it was found that long-term sequential therapy applying three drugs without drug holiday was overall the most promising strategy. Even though the strongest decrease in nuclei count was detected after G and Ox monotherapy, sequential application of Ox - G - 5-FU or Ox - 5-FU - G also led to a strong decrease of all Panc1 cell populations, with the strongest affection of Panc1 Holoclone cells. These data suggest that in Panc1 cell variants, especially in the CSC population, sequential therapy is at least partially dependent on the drug which is administered first, which was Ox for Panc1 cell lines, and on the treatment duration.

Comparing all sequential treatment strategies for Panc89 cell lines it could be found that short-term sequential therapy applying three drugs without drug holiday was the most effective treatment regimen. The strongest effects of short-term sequential therapies with three drugs and abundance of drug holiday could be determined for G monotherapy, as well as sequential application of G - 5-FU - Ox or G - Ox - 5-FU. Whereas the mentioned treatments affected parental Panc89 and Panc89 Paraclone cells by about 50-75%, Panc89 Holoclones were even stronger affected as the nuclei count was reduced to 20%. As already determined for Panc1 cells, these data suggest that sequential therapy also in Panc89 cells, especially in the CSC population, is dependent on the drug which is administered first, which was G for Panc89 cells, and on the treatment duration.

In summary, the success or failure of different therapy strategies seem to be highly dependent on the CSC/EMT phenotype of PDAC cell clones and heterogeneity of parental PDAC cells as

well as the applied treatment regimen, underscoring the consideration of CSC/EMT properties for decision making of cancer treatments.

5 Discussion

5.1 Basal characterization of parental Panc1 and Panc89, Holo- and Paraclone cells revealed distinct CSC-EMT phenotypes

Cancer cell plasticity and tumor heterogeneity, such as manifested by the presence and interplay of CSC and non-CSC populations, along with the pronounced tumor stroma are associated with poor prognosis and increased resistance to chemotherapy of PDAC^{130,138,289–291,139–146}. Besides this, CSCs and EMT are both linked to cancer progression and early metastasis, also in PDAC^{109,123–125}. Populations of CSCs are characterized by the unique ability to self-renewal, initiation of tumoral lesions at primary and secondary sites, the capability to resist cell death induction and to give rise to plastic, highly proliferative transit amplifying progenitor cell populations^{110–113,115–117,148}. As they also generate more or less differentiated cancer cells, CSCs are regarded as one important origin of tumor cell heterogeneity^{122,125–129}. The link between EMT and MET processes and CSCs has been already shown in different cancer entities^{109,123–125}, however, it is still poorly understood whether CSC and EMT phenotypes are always interconnected, how CSC phenotypes of epithelial and mesenchymal tumor cells are characterized and whether these might be related to different functional malignant outcomes. To gain a better understanding of plasticity in PDAC with a particular focus on CSCs and EMT and its contribution to malignancy associated properties, two different PDAC cell models were comparatively analyzed: Mesenchymal Panc1 cells are derived from a primary PDAC and have probably already undergone EMT, while Panc89 cells originate from a lymph node metastasis and have presumably undergone EMT and MET processes, having led to metastatic outgrowth in the lymph node²⁶⁶. Isolated Panc1 and Panc89 Holo- and Paraclone cells, as well as the two related parental cell populations, were comprehensively analyzed by expression and functional analysis *in vitro* and *in vivo*. First, CFAs and expression analysis of CSC-markers revealed different CSC phenotypes in the two PDAC cell lines. While parental Panc1 cells and Panc1 Holoclone cells were characterized by high Nestin expression and absent OVOL2 and SOX2 expression, Panc89 cells exhibited a partially inverse expression pattern showing an OVOL2-dominated phenotype for parental Panc89 cells and a SOX2-dominated phenotype for Panc89 Holoclone cells. CFAs showed a similar number of colonies formed by parental Panc1 and Panc89 cells, with a Holoclone-dominated colony formation of parental Panc89 cells and a Paraclone-dominated colony formation of parental Panc1 cells. This indicates stronger self-renewal potential for parental Panc89 cells compared to parental Panc1 cells. Moreover, the

CSC phenotype of Panc89 Holoclones was associated with a higher colony formation ability compared to Panc1 Holoclones, indicating a more pronounced self-renewal capacity of these cells. Furthermore, pathways involved in the activity of cell cycle checkpoints, transcriptional regulation of TP53 and Mueller Plurinet overall seemed to play a role in the PDAC CSC populations, as these genes were upregulated in both Panc1 and Panc89 Holoclone cells compared to related Paraclones. Mueller Plurinet is a protein-protein network shared by pluripotent cells and has become a prominent classifying system for pluripotency and self-renewal properties²⁸⁰. Activation of TP53 and cell cycle checkpoints are interconnected since TP53 enables dividing cells to repair anomalies before proceeding through cell cycle checkpoints, acting as a tumor suppressor²⁹²⁻²⁹⁵. Therefore, mutations in TP53 are strongly associated with tumor progression in various human cancers, also in PDAC, and recent reports have shown its impact on stemness properties in cancer cells^{266,292,301,302,293-300}. Overall, these data support the CSC phenotype of Panc1 and Panc89 Holoclones.

Comparing the colony formation ability of Paraclone cells from both Panc1 and Panc89 cells it was found that Panc89 Paraclone cells predominantly formed Holoclone colonies, while Panc1 Paraclone cells only gave rise to Paraclone colonies, indicating the ability of Panc89 Paraclone cells to gain self-renewal properties and being highly plastic. Furthermore, both Panc1 and Panc89 Paraclones showed activation of neutrophil degranulation. Neutrophils are first responders to inflammation, and it has been shown that neutrophil-like populations act as important players in cancer development and tumor progression. The degranulation of neutrophils is suggested to play a significant role in promoting the transport and extravasation of circulating tumor cells, thus, facilitating their metastatic migration and invasion^{303,304}.

Next, clear differences with respect to the EMT phenotype were noted between all cell variants. Parental Panc89 cells and Panc89 Holoclone cells were predominantly characterized by an epithelial phenotype exemplified by high E-cadherin expression and concomitant low expression of mesenchymal markers (Vimentin, L1CAM, ZEB1 and ZEB2). In contrast, all Panc1 cell variants were characterized by low E-cadherin expression and marked expression of mesenchymal markers underscoring their mesenchymal phenotype. Of note, Panc89 Paraclone cells seemed to exhibit a partial/hybrid EMT phenotype^{97,98,102,104} as they showed high E-cadherin expression, low Vimentin expression but also considerable expression of L1CAM, ZEB1 and ZEB2, which was higher compared to the other Panc89 cell variants but still lower compared to all Panc1 cell variants (despite ZEB2). These phenotypes were widely confirmed by

IHC staining of tumors formed *in vivo* by the inoculated Holo- or Paraclone cell populations of Panc1 and Panc89. However, in Panc89 Holoclone tumors areas with high L1CAM expression and low SOX2 expression but also with ZEB1 or ZEB2 expression were noted, and Panc1 Holoclone tumors showed elevated SOX2 expression which was absent in Panc1 Holoclone cells *in vitro*. This might be due to the fact that SOX2 functions as a stability factor in the control of PDAC cells, since SOX2 expression has been shown to be required for cell growth of PDAC cells ³⁰⁵. It was shown that SOX2 needs to be stably expressed, as increased as well as decreased levels of SOX2 reduces tumor growth of PDAC *in vitro* and *in vivo* ³⁰⁵. These findings underline our data, showing that a gain in SOX2 expression in Panc1 Holoclone tumors could be crucial to ensure tumor outgrowth *in vivo*. Altogether, these data indicate a phenotypic switch of the tumor cells *in vivo* presumably forced by the environmental factors the tumor cells are exposed to. A study on CRC showed that SOX2 expression in the tumor stroma is associated with invasion and worse prognosis of recurrence-free survival ³⁰⁶. Whether the tumor stroma of PDAC induces the expression of SOX2 in tumor cells or *vice versa* remains to be elucidated. Moreover, myofibroblasts and macrophages, both stroma cell populations which are significantly involved in pancreatic tumors and metastases ^{307,308}, were already shown to induce L1CAM expression in PDAC cells ^{95,309}.

Transcriptomic profiling of Panc1 and Panc89 cell variants revealed Panc89 cells to show features of both classical- and basal-like PDAC subtypes, with a stronger basal-like classification, while Panc1 cells showed no distinct subtype profile but weak classical-like characteristics. The classical subtype is defined by the presence of a higher differentiation status, better resectability and is associated with inflammation, while the basal subtype presents with poorer clinical outcome and loss of differentiation ²⁸. Based on this binary system, some studies subdivided the classical subtype, which represents the largest PDAC subgroup, into further subclasses ^{27–29,310–313}. Nevertheless, these well-established classifications seem to fail to reliably characterize PDAC due to the high tumor heterogeneity and cancer cell plasticity ^{310,311}, underscored by the above mentioned transcriptomic profiles of Panc1 and Panc89 cells which did not show a distinct classification.

As mentioned above, transcriptomic profiling of Panc1 and Panc89 Holoclone cells revealed similarities in both cell lines in *TP53* and cell cycle checkpoint regulation as well as stemness associated Mueller Plurinet activity. Nevertheless, two different CSC phenotypes could be identified: a mesenchymal Nestin-dominated (Panc1 Holoclone cells) and an epithelial SOX2-

dominated CSC phenotype (Panc89 Holoclone cells). Of note, both phenotypes were associated with clearly different functional properties. Besides the more pronounced colony formation, Panc89 Holoclone cells showed a clearly faster growth behavior compared to Panc89 Paraclone cells but also to Panc1 Holo- and Paraclone cells, being in line with the fact that proliferative activity of epithelial cells is generally higher than those of mesenchymal cells ^{37,50}. The finding that parental Panc1 cells showed faster cell growth compared to Panc1 Holo- and Paraclone cells suggest that a lack of clonal interactions in the isolated Holo- and Paraclone cell variants decreased the growth rates of these cells and may indicate that clonal interference may impair cell growth behavior of mesenchymal PDAC cells. Contrary, parental Panc89 cells and Panc89 Holoclone cells showed similar growth rates, while Panc89 Paraclones grew slower, possibly due to their mesenchymal characteristics.

Invasion assays revealed all Panc1 cell variants to exhibit a faster cell invasion in a typical mesenchymal manner, by single cell or chain-like invasion, with increased numbers of invasive fronts and longer invasion distances (doctoral thesis Arne Surrow, publication in progress). These findings are in line with the activity of EMT and invasion associated genes revealed by the transcriptomic analysis. In contrast, all Panc89 cell populations used an epithelial cluster mode of slow cell invasion by cell bulks with strong cell-cell contacts (doctoral thesis Arne Surrow, publication in progress), again fitting together with the epithelial phenotype of these cells ^{37,50,266}. Also in line with these findings, expression of Vimentin ^{81,83–87} and L1CAM ^{93–96} are associated with an increased invasive potential in a variety of cancers, also in PDAC cells. Of note, despite expression of Vimentin and L1CAM, Panc89 Paraclone cells did not show increased invasive potential, suggesting that a partial/hybrid EMT cell state does not necessarily favor invasion *in vitro*.

Moreover, these distinct growth and invasive properties were linked to different metastasis patterns *in vivo*. Panc1 Holoclone cells exhibited the strongest invasive potential using a mesenchymal invasion mode, being in line with the pronounced activation in cilium assembly revealed by transcriptomic analysis. This mesenchymal invasion of Panc1 Holoclones led to the highest number of microscopic metastatic lesions predominantly formed in the pancreas, compared to epithelial Panc89 cell variants and Panc1 Paraclones.

The fact that Panc1 Holoclone cells led to formation of more and larger tumors compared to Panc1 Paraclones underscores that the presence of CSC properties in mesenchymal PDAC cells is prerequisite for metastatic tumor formation. However, inoculation of Panc89 Holoclones

led to the most pronounced macroscopic tumor burden, with the highest number and the largest tumors compared to all other cell variants. These findings are in line with the increased Interferon signaling in Panc89 cell variants revealed by transcriptomic analysis, since Interferon signaling is associated with tumor growth and processing^{314–317}. Furthermore, this pronounced tumor burden of Panc89 cell variants was clearly associated with shorter survival times compared to Panc1 cell variants. However, differences between Panc89 Holo- and Paraclones were not that pronounced with respect to survival times, pointing again to a microenvironment mediated phenotypic switch of the tumor cells (see above). Noteworthy, tumor formation of Panc1 and Panc89 Paraclone cells suggested that not only Holoclone cell populations, but also non-CSC variants could gain CSC properties. Both Paraclone variants were able to induce tumor formation *in vivo*, although not as pronounced as Panc1 and Panc89 Holoclone cells. Panc89 Paraclone cells have already proven their self-renewal capacities by the pronounced formation of Holoclone colonies in the CFA, while Panc1 Paraclone cells only gave rise to Paraclone colonies. This gain of CSC properties of Panc1 and Panc89 Paraclone cells *in vivo* might also been mediated by the tumor microenvironment. These findings are in line with the current literature potentially indicating that CSC characteristics can be gained and lost depending on the tumor microenvironment^{125–129}. Nevertheless, expression of CSC-markers *in vivo* remained poor in both Panc1 and Panc89 Paraclone tumors.

In contrast to Panc1 tumors being mainly located in the pancreas, tumors formed by Panc89 cell variants predominantly manifested in the peritoneum. Adhesion assays to organ specific endothelial cells did not reveal any considerable differences between Holo- and Paraclone populations of either cell line but indicated that Panc89 cell variants adhere better to lung endothelium than Panc1 cell variants (doctoral theses Arne Surrow and Umut-Ulas Yesilyurt, publication in progress). However, these results were not in line with the tumor manifestation pattern observed *in vivo*, which might indicate that the different organ colonization and metastatic outgrowth of the inoculated cell populations rely more on other cell properties as well as the local microenvironment in the organ than adhesion to the organ specific endothelium. Moreover, CSC properties do not seem to determine homing to secondary sites rather than dissemination mode and outgrowth of the tumor cells.

As outlined before, both Nestin and SOX2 have been associated with CSC phenotypes and were linked to malignancy associated properties. Thus, shRNA mediated reduction of Nestin expression led to decreased tumor volume and hepatic metastases in a murine PDAC

model^{171,172}, being in line with our results demonstrating that Nestin expressing Panc1 Holoclone cells showed higher self-renewal capacity and invasive properties *in vitro* and formed higher number of tumors *in vivo* compared to Panc1 Paraclone cells. In contrast, Panc89 Holoclones lacked Nestin expression, but were characterized by high SOX2 expression compared to Panc89 Paraclone cells and to all Panc1 cell variants. However, *in vivo* Panc1 and Panc89 Holoclone tumors both exhibited similar amounts of SOX2, supporting the role of the micro-environment as determining factor of distinct CSC properties^{125–129,306}. Of note, elevated SOX2 expression in Panc89 Holoclones was associated with the formation of the highest number of colonies and the fastest growth rate *in vitro* and the formation of large tumors *in vivo*. Furthermore, it can be speculated that elevation of SOX2 might also be involved in outgrowth of Panc1 Holoclone tumors^{165,175,305}.

PDAC cells are known to predominantly metastasize to the liver, lung and the peritoneum^{6,19,20}. As described above, the highest number of Panc1 Holoclone tumors was predominantly found in the pancreas, while Panc89 Holoclone cells formed most tumors in the peritoneum.

In summary, these results support the view that mesenchymal CSC have a higher propensity to spread and the ability to colonize secondary sites (exemplified by the highest number of total microscopical tumoral lesions), which however does not ultimately lead to immediate disease progression and short survival. In contrast, an epithelial CSC phenotype seemed to be associated with slow cell invasion but the concomitant advantage for rapid tumor outgrowth, resulting in a massive increase of a life-threatening tumor burden (exemplified by the highest number of macroscopic tumors and shorter survival). The fact that mesenchymal Panc1 cells are derived from the primary tumor, together with their gene activity associated with EMT and invasion, underscores that these cells have presumably undergone EMT to leave the primary tumor. However, whether these cells would ever have been able to form metastases in this patient remains unsolved. Furthermore, it may explain why most Panc1 Holoclone tumors were found in the pancreas, indicating that these cells are still optimally adapted to their original tissue. In contrast, epithelial Panc89 cells originate from a lymph node metastasis, thus these cells have already proven their disseminating potential to leave the primary tumor (either after EMT using a mesenchymal invasion mode or while maintaining an epithelial/hybrid cell stage using a cluster-like mode) and to grow out as metastasis at a secondary site (e.g., after MET).

Overall, our data support the view that different tumor subtypes as well as CSC phenotypes exist in PDAC that are associated with distinct EMT/MET phenotypes of PDAC cells, essentially determining PDAC cell fate and function.

5.2 Distinct CSC/EMT phenotypes of mesenchymal and epithelial PDAC cell variants differ in their response to mono- and sequential therapy

After having identified distinct CSC/EMT phenotypes in Panc1 and Panc89 cell variants *in vitro* and *in vivo*, different treatment strategies should comparatively be tested on parental Panc1 and Panc89 cells, as well as Holo- and Paraclones. This aimed at getting a better understanding of how tumor cell heterogeneity affects treatment response. It is known that adjuvant monotherapy with Gemcitabine or 5-FU after surgical resection of PDAC improves overall survival of patients for about six months compared to resection alone^{186,187}. Moreover, adjuvant therapies with combination regimens like FOLFIRINOX^{188,189}, Gemcitabine treatment together with nab-Paclitaxel^{190–194} and, neoadjuvant therapies, combining chemotherapy with or without radiotherapy before surgical resection, have become more prominent^{195,196}. Nevertheless, in most PDAC patients therapeutic resistance occurs, leading to treatment failure and early deaths of the patients³⁴. Evolution of resistance against treatment in cancer cells can be caused by many factors such as individual's genetic make-up, drug metabolism pathways, epigenetic modifications, tumor microenvironmental factors, tumor microbiome, immune evasion or enhanced DNA repair and gene amplification^{215,318–320}.

The aim of this part of the study was to investigate whether the different PDAC cell variants respond to chemotherapy commonly used in the clinic and whether therapy regimens based on evolutionary biology principles lead to better therapeutic effects. It is known that sequential therapy of PDAC patients with different anti-tumor agents in high or low doses, e.g. treatment with Gemcitabine/nab-Paclitaxel or Leucovorin/Fluorouracil followed by FOLFIRINOX application, can improve therapeutic efficacy and reduce side effects^{229,232–238}. For this purpose, this work should provide an insight into the possible impact of epithelial and mesenchymal characteristics as well as the role of CSC properties on the response to different sequential treatment strategies.

For the analysis of different sequential treatment strategies, parental Panc1 and Panc89, as well as Holo- and Paraclone cells were treated with each Gemcitabine, Oxaliplatin or 5-FU in

monotherapy or in sequential therapies comprising two- or three of the cytostatic drugs. Furthermore, cell response analysis was performed at two time points (short-term 72 h, long-term 120 h). Additionally, every short- and long-term treatment strategy was tested with or without drug holiday. Even though previous studies reported a longer chemotherapy holiday in solid tumor treatments to enhance drug responses to re-treatments ^{198,221–224}, this work could demonstrate that, independent of short-or long-term sequential treatment, drug holiday attenuated treatment responses in all Panc1 and Panc89 cell populations. One exception was determined for Panc1 Holoclone cells, which appeared to respond better to treatment with drug holiday compared to parental Panc1 cells and Panc1 Paraclone cells. These findings suggest that drug holiday in mesenchymal CSCs could mediate sensitization to chemotherapy, possibly due to the outgrowth of treatment sensitive cell clones or caused by reactivation of metabolic processes and cell proliferation during the treatment pause. This in turn can sensitize the cells to the following treatments. Short-term sequential therapy using two drugs without drug holiday overall led to moderate effects in parental Panc1 and Panc89 cells as well as Panc1 and Panc89 Paraclone cells, while both Holoclone populations were less affected. The most effective treatment strategies appeared to be long-term treatment with three drugs without drug holiday for Panc1 cell variants and short-term sequential treatment using three drugs without drug holiday for Panc89 cell lines. The good response to long-term therapy with three drugs without drug holiday in Panc1 cell lines, which also led to a strong reduction of Panc1 Holoclone cells, could be due to their slowly proliferating mesenchymal phenotype, which makes a longer application of the substances necessary. Of note, the best treatment response of all Panc1 cell variants during long-term treatment could be detected after monotherapies with Gemcitabine and Oxaliplatin, with the best response to Gemcitabine for parental Panc1 cells and Oxaliplatin for Panc1 Holoclones. Long-term sequential treatment with three drugs without drug holiday revealed the most pronounced treatment effects in Panc1 cell variants when the cells were first treated with Oxaliplatin, independent of the application of Gemcitabine and 5-FU as second or third drug. For Panc89 cell lines the best effects could be detected after short-term treatment with three drugs and without drug holiday. Whereas all Panc89 cell variants were less affected by 5-FU monotreatment as well as every sequential therapy that started with 5-FU application, treatment with Gemcitabine increased the treatment response. Of note, parental Panc89 cells and Panc89 Paraclone cells responded less to Gemcitabine monotherapy or every sequential treatment started with Gemcitabine compared

to Panc89 Holoclones which were highly affected by this treatment. This could be due to the fact that Panc89 Holoclone cells gave rise to high numbers of transit amplifying progenitor cells, which are a promising target for chemotherapy due to their fast proliferative characteristics and their epithelial phenotype ²⁸, leading to the strong response to Gemcitabine. Panc89 Paraclone cells showed less response to Gemcitabine mono- and sequential therapy compared to Panc89 Holoclones, which could be explained by their partial/hybrid EMT cell state associated with strong plasticity, together with their slower cell growth due to their mesenchymal traits. Parental Panc89 cells might have the ability to adapt faster to different treatments due to their high tumor cell heterogeneity and plasticity, therefore, were less affected by Gemcitabine treatment. In summary, focusing on the heterogeneous parental cell populations, Panc89 cells showed better responses to the different therapies compared to Panc1 cells and, regarding sequential therapies, it was found that mesenchymal Panc1 cells showed a better response to treatments when Oxaliplatin was applied as first drug, whereas Panc89 cells showed stronger affection when cells were first treated with Gemcitabine.

Deregulated metabolism of cancer cells is a hallmark of cancer ³²¹, also in PDAC, and plays a crucial role in drug resistance. An important metabolic adaption of PDAC cells is their response to Gemcitabine by further enhancing oxidative pentose phosphate pathway to produce high levels of pyrimidine derivatives, like cytosine monophosphate, leading to saturation of PDAC cells with cytidylates. This directly competes with the phosphorylation and activation of Gemcitabine, resulting in drug resistance ^{215,322}. Furthermore, cells of the tumor microenvironment, like tumor-associated macrophages or cancer-associated fibroblasts, are also well-known mediators of therapeutic resistance in PDAC ^{213,214}. Patients with low macrophage composition in their tumors have shown to respond significantly better to Gemcitabine treatment compared to those patients with high macrophage burden ²¹⁴. Additionally, cancer-associated fibroblasts have the ability to release deoxycytidine and thereby, promoting Gemcitabine resistance ²¹³. Moreover, the microbiome composition and its impact on PDAC have become more prominent over the last years. Several studies have shown that bacterial compositions of the tumor microbiome in PDAC determines chemo-sensitivity as well as chemo-resistance and that eradication of bacteria which favor drug resistances of tumor cells could contribute to increased drug sensitivity, making the tumor microbiome a promising target in PDAC therapy ^{318,319,323,324}. A pivotal role in the development of chemoresistance in PDAC plays the transcription factor NFκB. It was shown that enhanced NFκB signaling can induce chemoresistance

to Gemcitabine as well as platinum-based agents, such as Oxaliplatin, Cisplatin or topoisomerase inhibitors, being part of the FOLFIRINOX regimen ³²⁵. Target genes of NFκB are cytokine encoding genes like *IL-1β* ^{326,327}, *IL2* ^{328–330}, *IL6* ^{331–334}, *IL8* ^{335,336}, *IL10* ³³⁷ and *TNF* (TNFα) ^{338–340}. Furthermore, increased NFκB activity can induce expression of the gene *ABCB9*, encoding for ABC-transporters promoting the efflux of nutrients and drug compounds ³⁴¹, and also induce the expression of the mesenchymal marker Vimentin ^{342,343}, thus, promoting maintenance of a mesenchymal phenotype which is in turn associated with drug resistance. During this work it was found that parental Panc89 cells as well as the derived Paraclones showed stronger cytokine secretion of IL-8, IL-6 and IL-10 compared to Panc89 Holoclone cells, and that release of these cytokines was overall lower in related Panc1 cell lines. TNFα secretion was only found in Panc1 Paraclones and parental Panc89 cells (Supplementary figure 2). These results suggested a higher activation status of the NFκB pathway in Panc89 cell variants. By findings of previous studies it would have been suggested that Panc1 cells show a higher basal NFκB activity ³⁴⁴, which could not be confirmed by this study. Lastly, NFκB signaling regulates survival as well as activation and differentiation properties of CSCs, which in turn may also contribute to treatment resistance ³²⁵.

Furthermore, it is known that the efficacy of platinum-based therapies in PDAC is limited ^{345–347}. DNA damage repair pathways, e.g. due to repair of DNA lesions formed by platinum compounds by the nucleotide excision repair pathway, play a crucial role in the resistance to platinum-based agents like Oxaliplatin ^{345–348}. Moreover, hepatocyte nuclear factor 1 homeobox A (HNF1A) ³⁴⁹, multidrug resistance protein 2 (MRP2, encoded by the *ABCC2* gene) ³⁵⁰ and the induction of oxidative stress mediated by platinum-based agents ³⁵¹ were recently found to mediate Oxaliplatin resistances in gastric cancer, CRC as well as PDAC ^{349–353}. Together with the findings of this work, these data suggest the named factors to be promising targets to determine resistance mechanisms against Oxaliplatin in PDAC cell variants, especially in Panc89 cell lines which responded worse to Oxaliplatin treatment compared to Panc1 cell variants.

Tumor cells are either initially resistant to treatment or resistance evolves during or by therapy ^{198,199}, further influenced by different subtypes of PDAC cells, like the well-established basal- or classical-like subgroups ^{27,29,310,312}. Acquired resistance can lead to induction of resistance mechanisms or to the accumulation of treatment-resistant cell clones caused by kill-

ing of the sensitive cell populations ¹⁹⁹. To prevent this, therapy concepts that consider principles of evolutionary biology, like adaptive or sequential therapies ^{226,227,229–231,234,354}, are thus promising treatment strategies.

Concerning the basal- and classical-like subtypes of PDAC cells, Moffitt and colleagues have demonstrated that patients with basal-like tumors showed a strong trend towards better responses to adjuvant therapy compared to patients burdened with tumors of the classical subtype ²⁸. These findings are in line with the data of this work, showing Panc89 cell variants to exhibit a basal-like phenotype, albeit genes of both basal- and classical-like subtypes co-existed, which is associated with an increased treatment response to different therapy strategies compared to Panc1 cell lines.

Based on this work, the following treatment strategies are proposed considering the CSC/EMT features of PDAC cells (Figure 34).

CSC phenotype	Nestin-dominated				SOX2-dominated			
EMT phenotype	Mesenchymal (Panc1)				Epithelial (Panc89)			
Therapy	Sequential therapy				Sequential therapy			
	Mono	2 drugs without DH	2 drugs with DH	3 drugs without DH	Mono	2 drugs without DH	2 drugs with DH	3 drugs without DH
Short-term (72 h)	Oxaliplatin Gemcitabine	Ox – G Ox – 5-FU	Ox – G Ox – 5-FU	×	Gemcitabine Oxaliplatin	G – 5-FU G – Ox	G – 5-FU G – Ox Ox – G	G – 5-FU – Ox G – Ox – 5-FU
Long-term (120 h)	Oxaliplatin Gemcitabine		×	Ox – G – 5-FU Ox – 5-FU – G	Gemcitabine		×	Ox – G – 5-FU G – Ox – 5-FU
Notes	- G better response in parental cells, - Ox better response in Holoclones - DH no effect (except for the better response of Holoclones)				- Strong effects of G on Holo- and Paraclones - DH no effect			

Figure 34 Proposed strategies for optimization of PDAC treatment considering CSC and EMT phenotypes. (Ox = Oxaliplatin, G = Gemcitabine, 5-FU = 5-Fluorouracil, DH = drug holiday, CSC = cancer stem cell, EMT = Epithelial-to-Mesenchymal-Transition, Mono = monotherapy)

In summary, the data of this work indicate that distinct CSC phenotypes in epithelial or mesenchymal PDAC cell variants are associated with clear differences in their functional characteristics like cell growth, metastatic properties and responses to therapy. In order to achieve an improvement in the therapy of PDAC patients, it is recommended to consider these different CSC/EMT phenotypes of PDAC cells for the selection of an appropriate treatment.

5.3 Conclusion and outlook

With a 5-year survival rate of about 12% and an ongoing increase in death rate, PDAC has one of the poorest prognoses of all malignant diseases ¹⁻³. One reason for this dismal situation is the early development of metastases, which often precede detection and diagnosis of primary PDAC. Therefore, the majority of patients are diagnosed at an advanced or even metastasized stage leaving only palliative treatment ⁵⁻⁷. The acquisition of CSC and EMT properties are regarded to be essential for tumor initiation at primary and secondary sites and to give rise to heterogenous, highly plastic cancer cell populations with the probability to resist cell death induction by chemotherapy ^{37,109,112,113,118-121}. These facts underscore the importance to improve early disease detection and treatment of PDAC. Therefore, this study aimed at getting a better understanding of heterogeneity and plasticity in PDAC and how identification of distinct CSC/EMT phenotypes could be implemented to improve therapy responses.

The evaluation of PDAC cells originating from a primary tumor (Panc1) revealed mesenchymal characteristics associated with a Nestin-dominated CSC phenotype, while PDAC cells originating from a lymph node metastasis (Panc89) were characterized by epithelial properties associated with a SOX2-dominated CSC phenotype. Analyzing isolated CSC (Holoclone) and non-CSC (Paraclone) populations from Panc1 and Panc89 cells, huge differences in the transcriptomic profile, in gene and protein expression patterns and functional characteristics of parental cell variants as well as Holo- and Paraclone cells were identified. Whereas Panc1 Holoclone cells were the only cell population of Panc1 cell lines with the ability to give rise to mostly Holoclone colonies, all Panc89 cell variants predominantly formed Holoclone colonies, reflecting the strong self-renewal properties of these cells. Panc1 Holoclones mostly differed from their parental cell line in the expression of ZEB2, while expression of the CSC-marker Nestin was similar, suggesting ZEB2 to be the crucial factor for maintenance of stemness characteristics in mesenchymal Panc1 cells. Panc1 Paraclone cells expressed a mesenchymal phenotype but showed no CSC traits, and reflected the differentiated characteristics of this cell population. Panc89 Holoclone cells were characterized by their co-expression of the CSC-markers OVOL2 and SOX2, whereas Panc89 Paraclone cells showed a partial/hybrid EMT phenotype presented by co-expression of epithelial and mesenchymal markers and the phenotypic stability factor OVOL2.

Differences in response to sequential therapies could be detected for Panc1 Holoclone cells, which in general were less affected by sequential treatments compared to parental Panc1 and

Panc1 Paraclone cells. Panc1 Holoclones showed stronger affection compared to Panc1 parental and Paraclone cells by sequential therapy when drug holiday was applied, while poorer response was detected after therapies without drug holiday. Contrary, for Panc89 cell lines it was found that Panc89 Holoclone cells responded better to most therapies compared to parental Panc89 and Panc89 Paraclone cells. One exception was long-term therapy with drug holiday, where cell numbers of Panc89 Holoclone cells were partially even increased compared to control and related parental and Paraclone cells.

In summary, it could be determined that Panc1 cells showed stronger affection by long-term treatments, while Panc89 cells responded better to short-term treatments. Nevertheless, in all cell populations it was found that the applied sequential therapies were mostly not as effective as monotherapy (with Gemcitabine for Panc89 cells, and Gemcitabine or Oxaliplatin for Panc1 cells). Based on these results it is recommended to extend the research on novel treatment strategies by modifying different parameters.

Regarding the CSC- and EMT-markers that were differently expressed in mesenchymal and epithelial PDAC cells, knockdown (KD) experiments would be of high interest to analyze whether silencing of distinct genes impacts the characterized phenotypes, along with their functional properties. Thus, after downregulation of Nestin and ZEB2 in Panc1 Holoclone cells, the impact on self-renewal properties, growth behavior, adhesion, migration and invasion properties as well as therapy response and tumorigenicity *in vitro* and *in vivo* should be investigated. Silencing of mesenchymal markers L1CAM and Vimentin in parental Panc1 cells and Panc1 Holoclones would be important to find whether the downregulation of these genes induces a change in their functional properties, like the adhesive behavior. For parental Panc89 cells gene silencing of OVOL2 could be tested to investigate the impact on colony formation ability and therefore, their self-renewing potential. SOX2 KD in Panc89 Holoclone cells should be analyzed for evaluating changes in their mesenchymal marker expression, as well as the impact on self-renewal properties, adhesion, migration, invasion, tumor progression and therapy response. Silencing of OVOL2 and L1CAM in single- and double-KDs in Panc89 Paraclone cells should be performed to analyze whether the cells lose their partial/hybrid EMT cell state or whether this phenotype is based on other factors. Furthermore, KD of OVOL2 and/or L1CAM might impact the therapy response of Panc89 Paraclones. Moreover, it is recommended to establish IFS and IHC for OVOL2 to investigate the protein expression *in vitro* and *in vivo*.

Regarding the response to therapy, different options should be further investigated. Since IC₅₀ represents the half maximal inhibitory concentration of an agent, it would be interesting to analyze whether PDAC cells undergo a stronger affection when drug concentrations are minimally increased. Moreover, as the performed sequential therapies were executed with only one drug at a time concomitantly, applying minimal doses of two or three drugs at the same time could also be promising, as combinational regimens already have become more prominent in clinical applications ^{188–194}. Since parental Panc89 cells have been shown to respond less to different treatment strategies compared to Panc89 Holo- and Paraclone cells, it was suggested that these results were subject to clonal interactions. To analyze whether clonal interaction occurs between CSCs and non-CSCs, it would be of interest to treat mixed cell populations with defined amounts of Holo- and Paraclone cells and to analyze how the treatment response changes in presence of a more CSC- or non-CSC-dominated population. Furthermore, applying agents with different modes of action would be of interest. For this purpose, combinational therapies with cytostatic drugs together with less toxic plant-based substances, like galactans, or antibody-drug conjugates, which have been shown to sensitize tumor cells to Gemcitabine in pancreatic cancer ^{355,356}, could be tested as well as the combination of chemotherapy and antibiotics. Previous studies identified that combined treatment of Gemcitabine and Doxycycline led to significantly more cell death of pancreatic cancer cells compared to Gemcitabine treatment alone, and that Doxycycline treatment *in vitro* showed cytotoxic effects on Panc1 cells ^{357,358}, being also of interest for microbiome-based treatments in PDAC ³²³. In addition, characterization of therapy surviving cells would be of great interest to evaluate which phenotypes of cells remain after different treatment strategies. It should be investigated whether there is a possibility to target the surviving cells with a different treatment regimen, as these cells would be the origin of re-outgrowth of the tumor cell population, which would further optimize this treatment strategy. Thus, it is recommended to characterize these surviving cells with respect to CSC/EMT characteristics.

In case of mesenchymal Panc1 cells it would be of interest to analyze whether the treatment response could be increased by reverting their mesenchymal phenotype towards epithelial-like cells, performed by stimulating the expression of CSC-markers that are associated with MET induction, like OVOL2. A previous study of our group reported that the blockade of TNF α promotes the reversion of mesenchymal characteristics in Panc1 cells towards more epithelial properties, reflected by decreased expression of ZEB1, Vimentin and L1CAM together with an

increased expression of E-cadherin¹⁸⁴. This MET-like process initiated by TNF α blockade could be a promising approach for further investigating novel treatment strategies. Moreover, it was found that inhibition of TNF α induced antitumoral effects and reduced metastasis³⁵⁹. Furthermore, it could be evaluated whether KD of distinct CSC-markers, like Nestin or SOX2, impacts therapy response in Panc1 and Panc89 cells. Finally, since this work focused on *in vitro* analysis to investigate the efficacy of different treatment strategies, it would be of interest to validate therapy responses *in vivo*, where tumor cells are exposed to the tumor stroma and microenvironmental factors.

Since the two analyzed PDAC cell lines do not sufficiently reflect the inter- and intratumor heterogeneity, further research is needed by characterizing larger panels of isolated CSC and non-CSC clones from other epithelial and mesenchymal PDAC cell lines and also freshly isolated tumor cells from primary tumor tissue. This would help to further identify distinct tumor subtypes and PDAC cell phenotypes with respect to CSC-EMT traits that can be considered for the use of the most effective treatment strategy.

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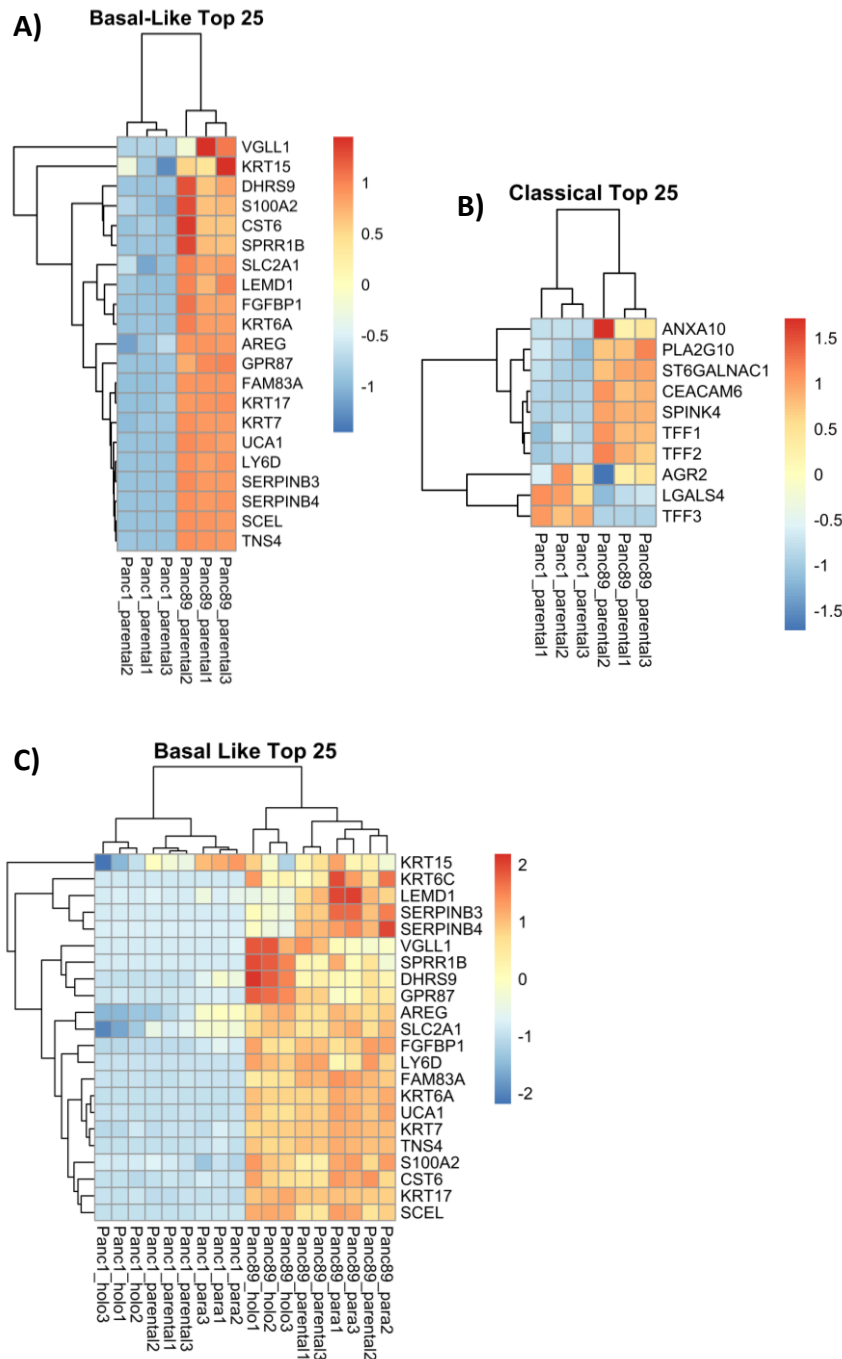
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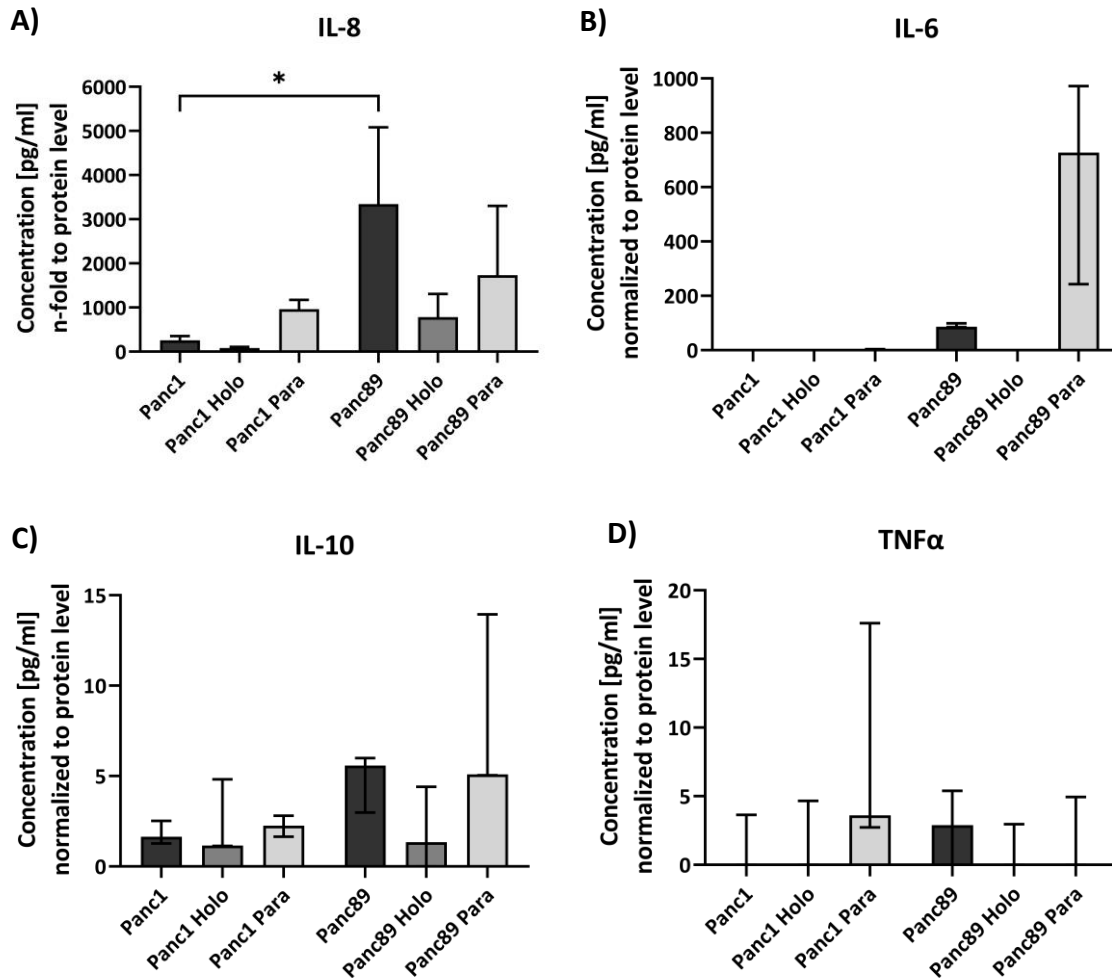
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Appendix

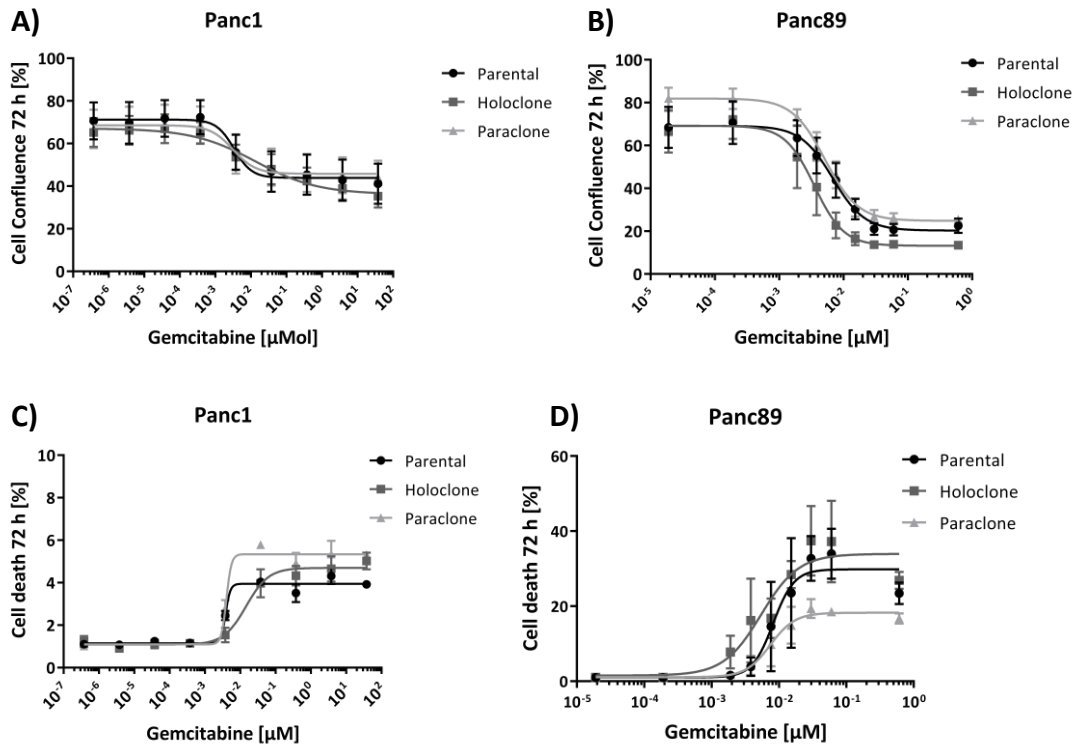
Supplementary data



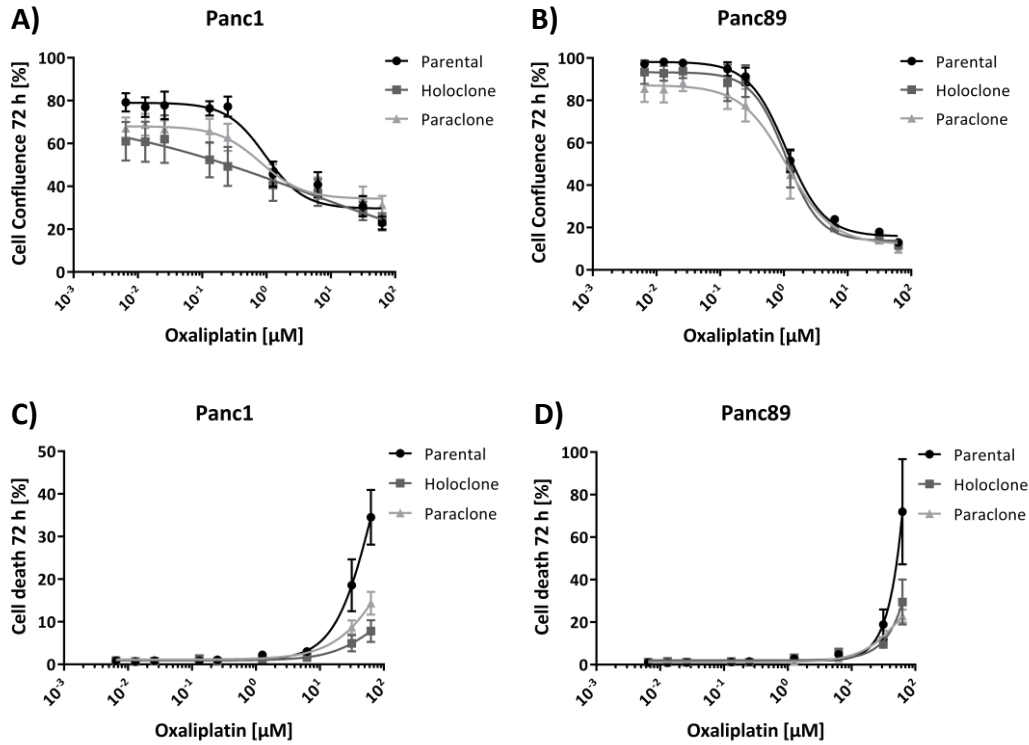
Supplementary figure 1 RNA sequencing-based subtyping of parental Panc1 and Panc89, Holo- and Paraclone cells. The subtyping analysis regarding B) classical-like and A, C) basal-like PDAC cell subtypes, based on the work of Moffit et al. ²⁸, revealed that Panc89 cell variants showed a strong tendency towards the basal-like subtype, but also classical-like characteristics. Panc1 cell variants showed no basal-like characteristics, but a tendency towards the classical subtype. Independent replicates n = 3.



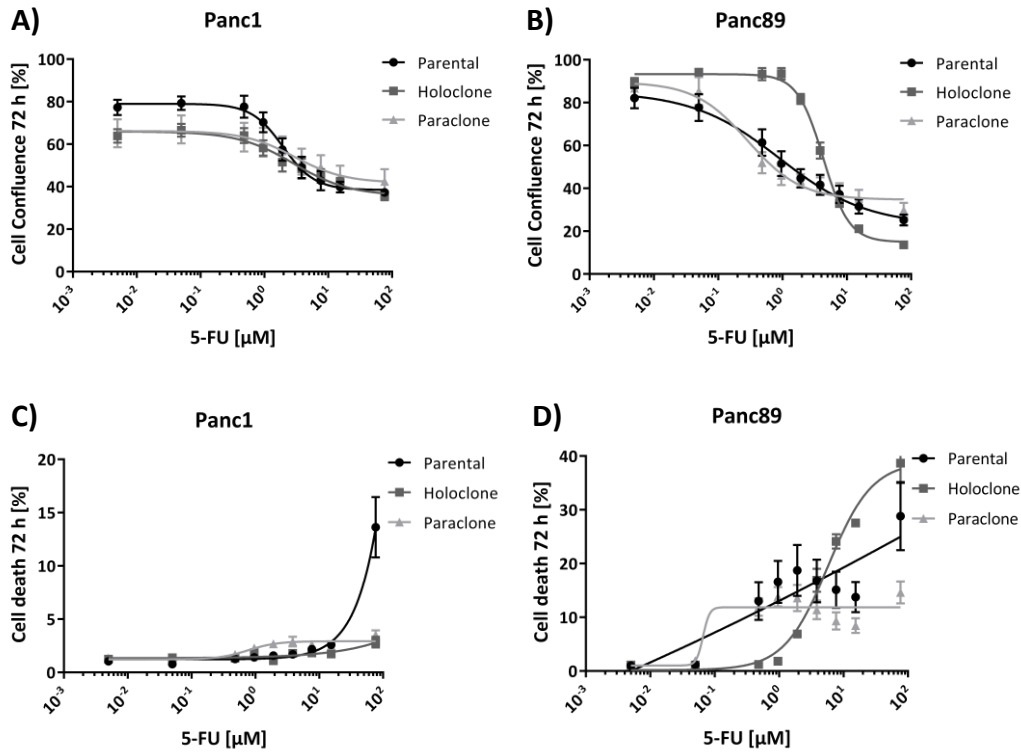
Supplementary figure 2 Different cytokine release in cell culture supernatants of parental Panc1 and Panc89, Holo- and Paraclone cells. Cell culture supernatants from parental Panc1 and Panc89, Holo- and Paraclone cells were analyzed for the abundance of cytokines *via* Multiplex analysis *via* LEGENDplex™ Multi-Analyte Flow Assay Kit. The graphs show the cytokine concentrations of A) IL-8, B) IL-6, C) IL-10, and D) TNFα in cell supernatants normalized to the protein concentration. Statistical analysis was performed by Shapiro-Wilk normality test, followed by ordinary one-way ANOVA for parametric data and Kruskal-Wallis test for nonparametric data. Parametric data are represented as mean with SD and non-parametric data as median with interquartile range. Supernatants of n = 3 independent experiments were analyzed. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$. (Panc1 = Panc1 parental, Panc89 = Panc89 parental, Holo = Holoclone, Para = Paraclone)



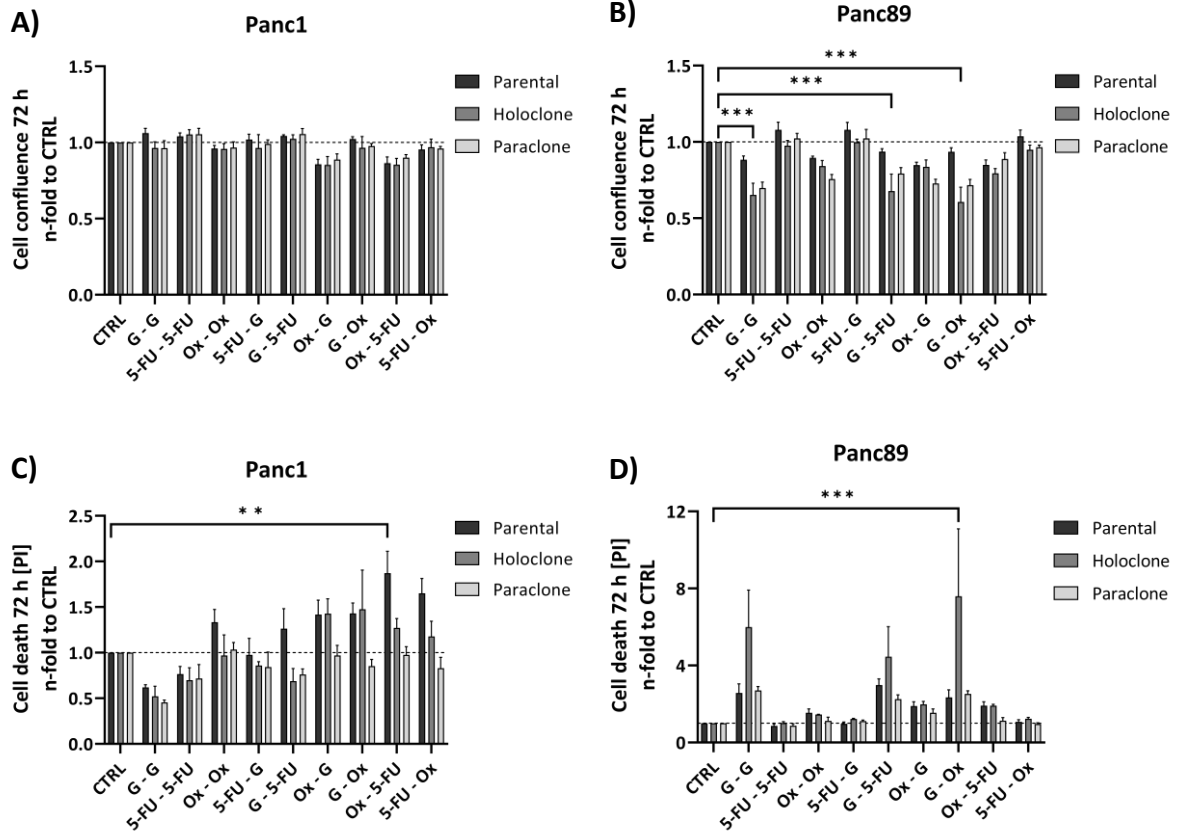
Supplementary figure 3 Dose-Response-Analysis for parental Panc1 and Panc89, Holo- and Paraclone cells for Gemcitabine. 0.5×10^4 of parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates. After 24 h, the cells were treated with nine different concentrations of Gemcitabine or NaCl control. After 72 h, an endpoint analysis was performed by measuring the cell confluence and the number of dead cells *via* PI staining for A, C) parental Panc1, Holo- and Paraclone cells, as well as B, D) parental Panc89, Holo- and Paraclone cells. Statistical analysis was performed by [Inhibitor] vs. response Nonlin-fit in GraphPad Prism. The graphs present the percentage of cell confluence (upper panel) and of dead cells (lower panel) after 72 h treatment. Independent experiments $n = 3 - 4$. (PI = Propidium iodide)



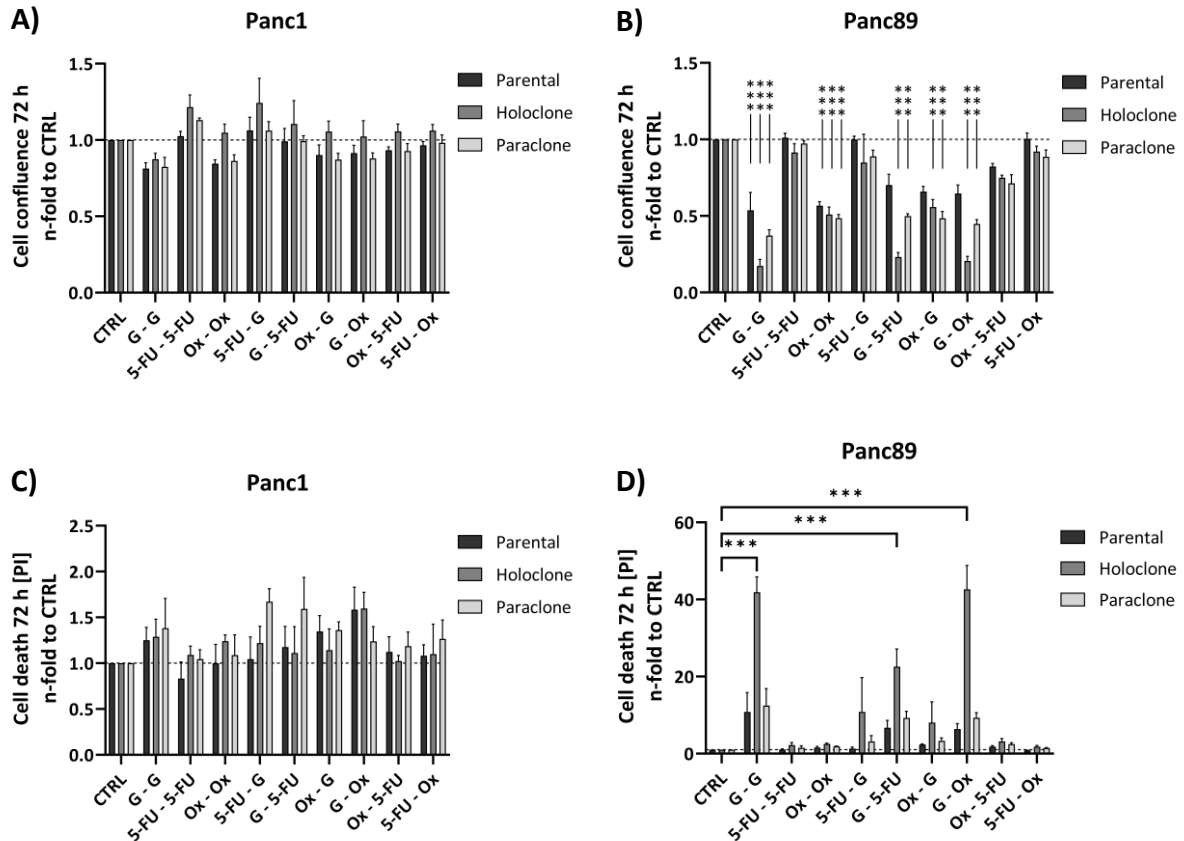
Supplementary figure 4 Dose-Response analysis (DRA) for parental Panc1 and Panc89, Holo- and Paraclone cells for Oxaliplatin. 0.5×10^4 of parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates. After 24 h, the cells were treated with nine different concentrations of Oxaliplatin or NaCl control. After 72 h, an endpoint analysis was performed by measuring the cell confluence and the number of dead cells *via* PI staining for A, C) parental Panc1, Holo- and Paraclone cells, as well as B, D) parental Panc89, Holo- and Paraclone cells. Statistical analysis was performed by [Inhibitor] vs. response Nonlin-fit in GraphPad Prism. The graphs present the percentage of cell confluence (upper panel) and of dead cells (lower panel) after 72 h treatment. Independent experiments $n = 3 - 4$. (PI = Propidium iodide)



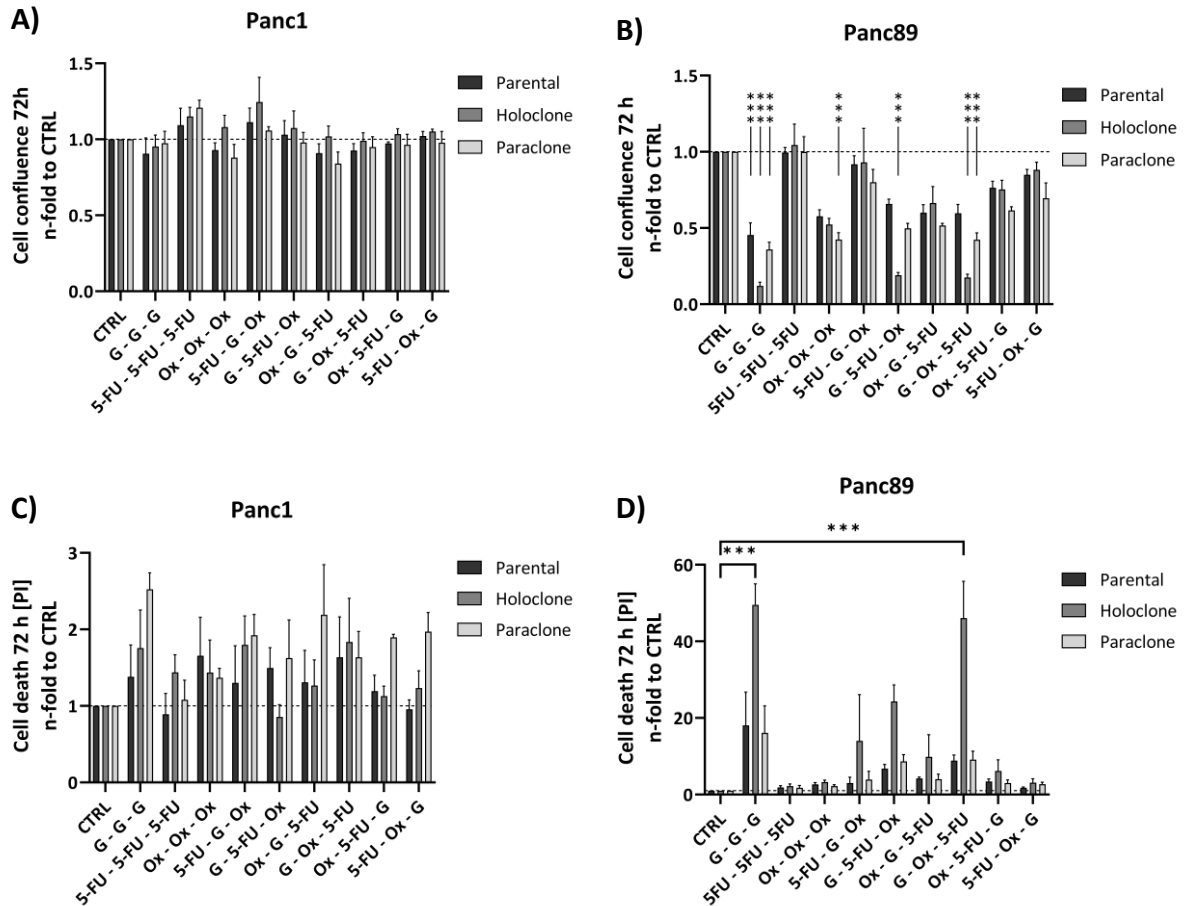
Supplementary figure 5 Dose-Response analysis (DRA) for parental Panc1 and Panc89, Holo- and Paraclone cells for 5-Fluorouracil (5-FU). 0.5×10^4 of parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates. After 24 h, the cells were treated with nine different concentrations of 5-FU or NaCl control. After 72 h, an endpoint analysis was performed by measuring the cell confluence and the number of dead cells *via* PI staining for A, C) parental Panc1, Holo- and Paraclone cells, as well as B, D) parental Panc89, Holo- and Paraclone cells. Statistical analysis was performed by [Inhibitor] vs. response Nonlin-fit in GraphPad Prism. The graphs present the percentage of cell confluence (upper panel) and of dead cells (lower panel) after 72 h treatment. Independent experiments $n = 3 - 4$. (PI = Propidium iodide, 5-FU = 5-Fluorouracil)



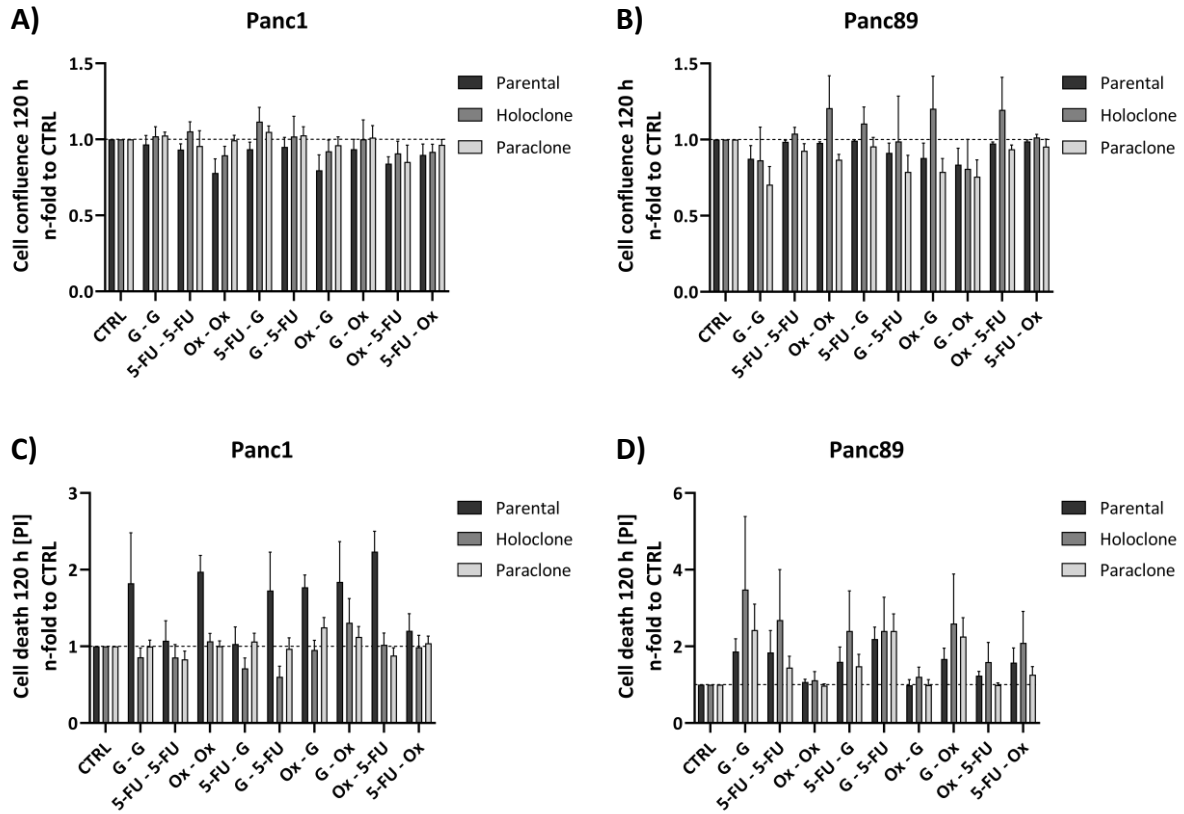
Supplementary figure 6 Short-term sequential therapy for 72 h with drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs were replaced with fresh cell culture medium for 24 h drug holiday. After drug holiday, the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as cell staining with Propidium iodide (for detection of dead cells) for A, C) parental Panc1, Holo- and Paraclone cells as well as B, D) parental Panc89, Holo- and Paraclone cells. The graphs show the cell confluence and cell death (PI) normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, $n = 4$. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5 FU = 5 Fluorouracil)



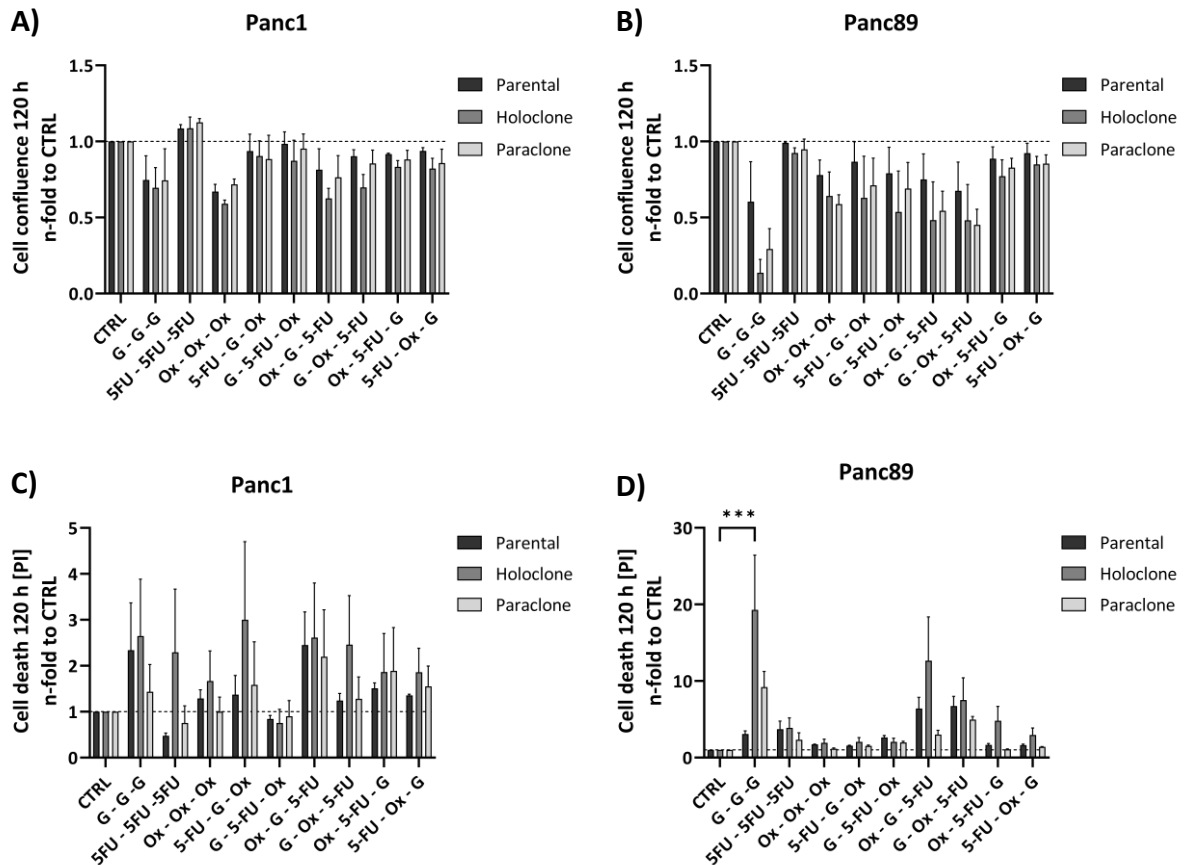
Supplementary figure 7 Short-term sequential therapy for 72 h without drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs were replaced with the second cytostatic drug (G, Ox or 5-FU) for 48 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as cell staining with Propidium iodide (for detection of dead cells) for A, C) parental Panc1, Holo- and Paraclone cells as well as B, D) parental Panc89, Holo- and Paraclone cells. The graphs show the cell confluence and cell death normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, $n = 3$. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5 FU = 5 Fluorouracil)



Supplementary figure 8 Short-term sequential therapy for 72 h with 3 cytostatic drugs without drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs were replaced with the second cytostatic drug (G, Ox or 5-FU) for another 24 h. Finally, the cells were treated with the third cytostatic drug (G, Ox or 5-FU) for additional 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as cell staining with Propidium iodide (for detection of dead cells) for A, C) parental Panc1, Holo- and Paraclone cells as well as B, D) parental Panc89, Holo- and Paraclone cells. The graphs show the cell confluence and cell death normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, $n = 3$. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5 FU = 5 Fluorouracil)



Supplementary figure 9 Long-term sequential therapy for 120 h with drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.25×10⁴ parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 72 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 72 h, the drugs were replaced with fresh cell culture medium for 24 h drug holiday. After drug holiday, the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 24 h. After a total therapy time of 120 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as cell staining with Propidium iodide (for detection of dead cells) for A, C) parental Panc1, Holo- and Paraclone cells as well as B, D) parental Panc89, Holo- and Paraclone cells. The graphs show the cell confluence and cell death normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, n = 5. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5 FU = 5 Fluorouracil)



Supplementary figure 10 Long-term sequential therapy for 120 h in use of 3 cytostatic drugs without drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.25×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs were replaced with the second cytostatic drug (G, Ox or 5-FU) for 48 h. Finally, the cells were treated with the third cytostatic drug (G, Ox or 5-FU) for additional 48 h. After a total therapy time of 120 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as cell staining with Propidium iodide (for detection of dead cells) for A, C) parental Panc1, Holo- and Paraclone cells as well as B, D) parental Panc89, Holo- and Paraclone cells. The graphs show the cell confluence and cell death normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, $n = 3$. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5 FU = 5 Fluorouracil)

List of Figures

- Figure 1 Progression of pancreatic ductal adenocarcinoma (PDAC) arising from pancreatic intraepithelial neoplasia (PanINs) is a multi-step process. The figure shows a schematic illustration of the progression from normal pancreatic ductal epithelium to low-grade and high-grade dysplastic PanINs and finally to (invasive) PDAC. Epithelial stages as well as genetic alterations during PDAC progression are shown. Low-grade PanINs bear early somatic changes in the *Kirsten rat sarcoma viral oncogene homologue (KRAS)* oncogene, while high grade PanINs can show inactivation of the *tumor suppressor gene p53 (TP53)* or underlying gene defects like *cyclin-dependent kinase inhibitor 2A (CDKN2A)*-, *small mother against decapentaplegic-family 4 (SMAD4)*-member or hereditary *Breast Cancer 2 (BRCA2)* mutations. Invasive PDAC shows more structural variations and genomic instability. Adapted from ^{10,18,24}. (ECM = Extracellular matrix)..... 3
- Figure 2 The metastatic cascade. This simplified illustration shows the multi-step process of metastasis, beginning with 1) the acquisition of a motile and invasive tumor cell phenotype followed by 2) the dissemination of tumor cells from the primary tumor, 3) intravasation of tumor cells into blood and lymphatic vessels, 4) survival of circulating tumor cells, 5) extravasation of tumor cells from the lumina of different vessels into the parenchyma of distant tissues and ending with 6) reversion of the motile phenotype, colonization of tumor cells, followed by proliferation and outgrowth of visible metastases at secondary sites. Created with BioRender.com. 6
- Figure 3 Simplified schematic illustration of cancer cell transition during Epithelial-to-Mesenchymal-Transition (EMT) and the reverse Mesenchymal-to-Epithelial-Transition (MET) process. The acquisition of an EMT phenotype enables cancer cells to disseminate, invade and form metastases at secondary sites. MET is associated with the reversion of invasive phenotypes, together with regaining proliferation capacity. In case of PDAC, ductal epithelial cells from primary sites lose their polarized, non-motile and highly proliferative phenotype and in turn, gain migratory and contractile characteristics, together with a loss of cell-cell adhesions and decreased proliferative potential. At secondary sites the motile, invasive characteristics are reversed to a highly proliferative, polarized phenotype leading the outgrowth of metastatic lesions. Created with BioRender.com. 7
- Figure 4 Representative Colony-Formation-Assay images of colonies formed by parental Panc1 and Panc89 cells and there of derived Holo- and Paraclone cells. Parental Panc1 and Panc89 as well as Holo- and Paraclone cells were analyzed regarding their colony-formation ability, being indicative for the cellular self-renewal properties, *via* Colony-Formation-Assay (CFA). Holoclones consisted of tightly, homogenously clustered cells with a regular boundary, while Paraclones consisted of dispersed, larger cells with an irregular boundary. Parental cells are characterized by a heterogeneous cell population comprising both Holo- and Paraclones. Scale bar = 100 μ m..... 43
- Figure 5 Parental Panc1 and Panc89 as well as their Holo- and Paraclone cells show different colony formation ability. Parental Panc1 and Panc89 cells and their Holo- and Paraclone cells were analyzed regarding their colony-formation ability *via* Colony-Formation-Assay (CFA). 400 cells of each cell line were seeded in duplicates/triplicates in 6-well plates and after eight to eleven days, the colonies were fixed with PFA and stained with crystal violet. The colonies formed were morphologically characterized regarding Holo- and Paraclones. Number of formed Holo- and Paraclones per well are shown for A) parental Panc1, Holo- and Paraclone cells as well as B) parental Panc89, Holo- and Paraclone cells. Y-axis in B) is segmented with 0-18 for the bottom part and 30-200 for the upper part. Data were analyzed with two-way ANOVA and are shown with standard error of means. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$ (independent experiments $n = 3-7$). 44

- Figure 6 Principal Component Analysis based on the 10% most varying transcripts across all of parental Panc1 and Panc89, Holo- and Paraclone cell samples using the R package PCAtools (v2.10.0). Principal Component Analysis (PCA) was performed for the highest variation in the first principal component (PC1) on the X-axis, and for the lower variations by the second principal component (PC2) on the Y-axis. All cell variants are denoted by color (red = Panc1 cell variants, blue = Panc89 cell variants) and symbol, respectively. Independent replicates n = 3. 46
- Figure 7 Gene Set Enrichment Analysis on the differential effect sizes on the reactome pathways in parental Panc1 and Panc89 as well as Holo- and Paraclone cell samples. Comparative Gene Set Enrichment Analysis (GSEAs) for A) parental Panc1 cells vs parental Panc89 cells, B) Panc1 Holoclones vs Paraclones and C) Panc89 Holoclones vs Paraclones are shown. GSEA on the shrunken log₂ fold changes from apeglm²⁷⁴ was performed using GAGE (v2.48.0)²⁷⁵ against Hallmark, Reactome and Gene Ontology Biological Processes (GOBP) gene sets extracted from the msigdb R package (v7.5.1). Independent replicates n = 3. 49
- Figure 8 Gene Set Variation Analysis of gene sets associated with positive or negative regulation on the EMT program, stemness and invasion properties in parental Panc1 and Panc89, Holo- and Paraclone cells. Gene sets from CancerSEA²⁸¹ were used to investigate whether Panc1 and Panc89 cell variants showed different activation of genes that are strongly associated with stemness (by Mueller Plurinet gene set), EMT and cell invasion. The numbering of all cell variants represents the number of the independent replicate (independent replicates n = 3). (EMT = Epithelial-to-Mesenchymal-Transition, holo = Holoclone, para = Paraclone) 50
- Figure 9 Transcriptomic marker gene analysis of parental Panc1 and Panc89, Holo- and Paraclone cells. Transcriptomic analysis revealed different expression of CSC- and EMT- associated marker genes in parental Panc1 and Panc89 as well as Holo- and Paraclone cells. *NES*, *OVOL2* and *SOX2* represented CSC-marker genes, *CDH1* (epithelial), *VIM* and *L1CAM* (mesenchymal) represented EMT-marker genes and *ZEB1* and *ZEB2* represented marker genes for EMT-induction. Y-axis shows the transcripts per million reads, X-axis shows analyzed genes. Data are represented as means with SD. Independent experiments n = 3. (CSC = Cancer stem cell, EMT = Epithelial-to-Mesenchymal-Transition) 52
- Figure 10 Gene expression analysis of EMT- and CSC-associated genes in parental Panc1 and Panc89, Holo- and Paraclone cells *via* quantitative real-time polymerase chain reaction. RNA of basal parental Panc1 and Panc89 as well as Holo- and Paraclone cells was isolated and transcribed into cDNA, which was used for quantitative real-time polymerase chain reaction (qPCR) analysis. Expression of the GOI was normalized to the expression of the reference gene GAPDH, and is thus shown as relative gene expression. The stemness marker A) *NES*, B) *SOX2* and C) *OVOL2* were selected as CSC panel. D) *CDH1*, E) *L1CAM* and F) *VIM* were chosen as EMT panel, and G) *ZEB1* and H) *ZEB2* were elected to represent EMT-inducers. Y-axis of *VIM* and *ZEB2* graphs were segmented to provide accurate representation of data. Parametric relative gene expression data were analyzed *via* one-way ANOVA and Tukey's multiple comparison with data representation as mean with SD. Kruskal-Wallis analysis and Dunn's multiple comparison test was used for nonparametric data and are represented as median with interquartile range. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$, independent replicates n = 3. (SD = Standard deviation, Holo = Holoclone, Para = Paraclone) 55
- Figure 11 Immunofluorescent staining of EMT- and CSC-markers in parental Panc1 and Panc89, Holo- and Paraclone cells. Cells were seeded on coverslips and for Panc1 cell lines the A) CSC-marker NESTIN and the EMT-inducer ZEB2 were stained, while B) the CSC-marker SOX2 and the mesenchymal EMT-marker L1CAM were stained in Panc89 cell lines. Additionally, all cell nuclei were stained with Hoechst33258 (Hoechst). Representative images from n = 3 independent experiments are shown. Scale bar = 100 μ m. 57

- Figure 12 Growth rate analysis of parental Panc1 and Panc89, as well as Holo- and Paraclone cells revealed Panc89 cell lines to show faster cell growth compared to Panc1 cell lines. 0.5×10^4 cells of each cell line were seeded in a 96-well plate in triplicates and cell confluence as well as nuclei number were monitored for 168 h. Logistic growth rate analysis *via* determination of cell confluence for A, C) parental Panc1 and Panc89, Holo- and paraclone cells and logistic growth rate analysis *via* determination of nuclei count for B, D) parental Panc1 and Panc89, Holo- and Paraclone cells are shown. Data are depicted as percent [%] cell confluence and total number [#] of nuclei. Analysis of k for cell confluence and nuclei count was performed by logistic growth analysis *via* Nonlin-fit in GraphPad Prism. E) Mean growth rates calculated from cell confluence and nuclei count analysis. Graphs present mean with SEM of n = 3 independent experiments. 60
- Figure 13 Shorter median survival of animals inoculated with Panc89 Holo- or Paraclone cells. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line). Y-axis shows the percent survival of mice, X-axis presents the POD when the animals were sacrificed because of reduced health status due to tumor burden or to terminate the experiment. Statistical analysis was performed by Kaplan-Meier-Survival analysis with curve comparison and Mantel-Cox log-rank test, resulting in significantly different survival curves with a P-value of ≤ 0.001 (***) . (Holo = Holoclone cells, Para = Paraclone cells, POD = post-operative day). 61
- Figure 14 Macroscopic tumor areas and organs of macroscopic tumor manifestation in Panc1 or Panc89 Holo- or Paraclone cell inoculated mice. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line). Organs and tumor formation were monitored *via* ultrasound until experiment termination. A, B) macroscopic tumor areas [mm^3] of Panc1 or Panc89 Holo- or Paraclone tumors as well as C, D) number of macroscopic tumor formation in different organs is shown. Statistical analysis was performed using A, B) unpaired t-test for non-parametric data and Mann-Whitney test and C, D) parts of a whole analysis. (Holo = Holoclone cells, Para = Paraclone cells)..... 64
- Figure 15 *Post mortem* analysis of microscopic tumors and microscopic tumor area in tissues of Panc1 or Panc89 Holo- or Paraclone cell inoculated animals. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line). Organs and tumor formation of stained tissue sections were microscopically analyzed regarding number and location of microscopic tumors as well as microscopic tumor areas [mm^2]. A, B) organs of microscopic tumor formation are represented as parts of a whole. C, D) analysis of microscopic tumor areas of Panc1 or Panc89 Holo- or Paraclone tumors was performed with unpaired t-test for non-parametric data and Mann-Whitney test. E) shows representative images of cyst formation in two independent tumors formed by Panc89 Paraclone cells. (Other = brown fat tissue, duodenum, undefinable; Holo = Holoclone cells, Para = Paraclone cells)..... 66
- Figure 16 Immunohistochemical analysis of Pan-Cytokeratin in tumor lesions of Panc1 or Panc89 Holo- or Paraclone cell inoculated mice. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line) and extracted tissues and tumor lesions were stained for Pan-Cytokeratin. Scale bars: left = 500 μm ; right = 100 μm 68
- Figure 17 Definition of tumor score by frequency (number of positive cells) and intensity (intensity of positively stained cells) for quantification of immunohistochemical staining of tumor sections from Panc1 and Panc89 Holo- and Paraclone cells *in vivo*. Frequency score was set from 1-4, intensity Score was set from 1-3. The figure shows exemplary pictures of stained tumor tissues for frequency- and intensity score definition. Scale bar = 100 μm 69
- Figure 18 Immunohistochemical analysis of EMT-markers in tumor lesions of mice inoculated with Panc1 or Panc89 Holo- or Paraclone cells. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line) and extracted tissues and tumor lesions were stained for the epithelial marker A) E-cadherin and the mesenchymal markers B) L1CAM and

C) Vimentin. Representative pictures of stained tissues of Panc1 and Panc89 Holo- or Paraclone tumors are presented in the left and middle picture panels, the right panel shows the statistical analysis of Frequency and Intensity score of positively stained cells. The upper row shows the data of analyzed Panc1 Holo- and Paraclone lesions, the lower panel shows the data of Panc89 Holo- and Paraclone lesions. Statistical analysis was performed by Shapiro-Wilk normality test, followed by ordinary one-way ANOVA plus Tukey's multiple comparison test for parametric data and Kruskal-Wallis test plus Dunn's multiple comparison test for nonparametric data. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$. Scale bar = 100 μm 71

Figure 19 Immunohistochemical analysis of EMT-inducers in tumor lesions of mice inoculated with Panc1 or Panc89 Holo- or Paraclone cells. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line) and extracted tissues and tumor lesions were stained for the EMT-inducers A) ZEB1 and B) ZEB2. Representative pictures of stained tissues of Panc1 and Panc89 Holo- or Paraclone tumors are presented in the left and middle picture panels, the right panel shows the statistical analysis of Frequency and Intensity score of positively stained cells. The upper row shows the data of analyzed Panc1 Holo- and Paraclone lesions, the lower panel shows the data of Panc89 Holo- and Paraclone lesions. Statistical analysis was performed by Shapiro-Wilk normality test, followed by ordinary one-way ANOVA plus Tukey's multiple comparison test for parametric data and Kruskal-Wallis test plus Dunn's multiple comparison test for nonparametric data. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$. Scale bar = 100 μm 72

Figure 20 Immunohistochemical analysis of CSC-markers in tumor lesions of mice inoculated with Panc1 or Panc89 Holo- or Paraclone cells. Mice were inoculated intrasplenically with 1×10^4 Panc1 or Panc89 Holo- or Paraclone cells (group of ten mice per cell line) and extracted tissues and tumor lesions were stained for the CSC-markers A) Nestin and B) SOX2. Representative pictures of stained tissues of Panc1 and Panc89 Holo- or Paraclone tumors are presented in the left and middle picture panels, the right panel shows the statistical analysis of Frequency and Intensity score of positively stained cells. The upper row shows the data of analyzed Panc1 Holo- and Paraclone lesions, the lower panel shows the data of Panc89 Holo- and Paraclone lesions. Statistical analysis was performed by Shapiro-Wilk normality test, followed by ordinary one-way ANOVA plus Tukey's multiple comparison test for parametric data and Kruskal-Wallis test plus Dunn's multiple comparison test for nonparametric data. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$. Scale bar = 100 μm 74

Figure 21 Immunohistochemical analysis of Panc89 Holoclone tumors that underwent a plasticity related switch *in vivo*. Mice were inoculated intrasplenically with 1×10^4 Panc89 Holoclone cells and L1CAM as well as SOX2 positive tumor lesions were analyzed. Comparative analysis of L1CAM and SOX2 expression in A) all Panc89 Holoclone tumors and B) of L1CAM expressing tumors are shown. Tumors formed by Panc89 Holoclone cells revealed a significant plasticity switch in 4/17 tumors, by downregulation of the CSC-marker SOX2 together with an increased expression of the mesenchymal marker L1CAM. Data were tested for normal distribution and afterwards Mann-Whitney test for non-parametric data was performed. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$ 75

Figure 22 Schematic representation of the experimental procedure for determining the half maximal inhibitory concentrations of the cytostatic drugs Gemcitabine, Oxaliplatin and 5-Fluorouracil. 0.5×10^4 of parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates. After 24 h, the cells were treated with nine different concentrations of either cytostatic drug or the carrier solution (NaCl) as control (CTRL) for 72 h. Afterwards, the amount of cell nuclei was analyzed by Hoechst33342 staining. The nuclei count analysis was used to determine the IC50 values by [Inhibitor] vs. response - Nonlin-fit in GraphPad Prism. Created with BioRender.com. 77

Figure 23 Determination of the half maximal inhibitory concentration for parental Panc1 and Panc89, Holo- and Paraclone cells for the cytostatic drugs Gemcitabine, Oxaliplatin and 5-Fluorouracil. For maximal

inhibitory concentration (IC₅₀) determination 0.5×10^4 of each parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates. After 24 h, the cells were treated with nine different concentrations of cytostatic drugs or were left treated with NaCl as CTRL. Afterwards, the cell confluence was monitored every 24 h. Finally, after 72 h of stimulation, an endpoint analysis was performed by measuring the amount of cell nuclei by Hoechst33342 staining. The nuclei count analysis was used to determine the IC₅₀ values by [Inhibitor] vs. response Nonlin-fit in GraphPad Prism. The graphs present the percentage of nuclei count after 72 h treatment normalized to NaCl control (Y-axis) and used concentration of respective cytostatic agents in μM (X-axis). IC₅₀ determination for A, B) Gemcitabine, C, D) Oxaliplatin and E, F) 5-FU for A, C, E) parental Panc1, Holo- and Paraclone cells as well as B, D, F) parental Panc89, Holo- and Paraclone cells. Independent experiments $n = 3 - 4$ 79

Figure 24 Illustration of short-term sequential therapy for 72 h without drug holiday. 0.5×10^4 Panc1 and Panc89 parental, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated with the first cytostatic drug (G, Ox or 5-FU) for 24 h or left untreated as CTRL. After 24 h, the drugs were removed and after a washing step the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 48 h. Cell confluence was monitored every 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as Propidium iodide (for detection of dead cells) and Hoechst33342 (for detection of cell nuclei) staining. The statistical evaluation was performed with the nuclei count data *via* Hoechst33342 staining. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil). Created with BioRender.com. 80

Figure 25 Short-term sequential therapy for 72 h without drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs were replaced with the second cytostatic drug (G, Ox or 5-FU) for 48 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by Hoechst33342 (for detection of cell nuclei) staining for A) parental Panc1, Holo- and Paraclone cells as well as B) parental Panc89, Holo- and Paraclone cells. The graphs show the percentage of analyzed nuclei count normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, independent experiments $n = 3$. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil) 81

Figure 26 Illustration of short-term sequential therapy for 72 h with drug holiday. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated with the first cytostatic drug (G, Ox or 5-FU) for 24 h or left untreated as CTRL. After 24 h, the drugs were removed and after a washing step replaced by fresh cell culture medium for a 24 h drug holiday. After drug holiday, medium was removed and the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 24 h. Cell confluence was monitored every 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as cell staining with Propidium iodide (for detection of dead cells) and Hoechst33342 (for detection of cell nuclei). The statistical analysis was performed with the nuclei count data *via* Hoechst33342 staining. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil). Created with BioRender.com. 83

Figure 27 Short-term sequential therapy for 72 h with drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs

were replaced with fresh cell culture medium for 24 h drug holiday. After drug holiday, the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by Hoechst33342 (for detection of cell nuclei) staining for A) parental Panc1, Holo- and Paraclone cells as well as B) parental Panc89, Holo- and Paraclone cells. The graphs show the percentage of analyzed nuclei count normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, independent experiments n = 4. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil)..... 84

Figure 28 Illustration of short-term sequential therapy for 72 h without drug holiday and use of three cytostatic drugs. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated with the first cytostatic drug (G, Ox or 5-FU) for 24 h or left untreated as CTRL. After 24 h, the drugs were removed and after a washing step the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for another 24 h. Afterwards, the cells were washed again and finally treated with the third cytostatic drug (G, Ox or 5-FU) for additional 24 h. Cell confluence was monitored every 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by again measuring the cell confluence as well as Propidium iodide (for detection of dead cells) and Hoechst33342 (for detection of cell nuclei) staining. The statistical evaluation was performed with the nuclei count data *via* Hoechst33342 staining. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil). Created with BioRender.com. 86

Figure 29 Short-term sequential therapy for 72 h with 3 cytostatic drugs without drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs were replaced with the second cytostatic drug (G, Ox or 5-FU) for another 24 h. Finally, the cells were treated with the third cytostatic drug (G, Ox or 5-FU) for additional 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by Hoechst33342 (for detection of cell nuclei) staining for A) parental Panc1, Holo- and Paraclone cells as well as B) parental Panc89, Holo- and Paraclone cells. The graphs show the percentage of analyzed nuclei count normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, independent experiments n = 3. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil) 87

Figure 30 Illustration of long-term sequential therapy for 120 h with three cytostatic drugs without drug holiday. 0.25×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated with the first cytostatic drug (G, Ox or 5-FU) for 24 h or left untreated as CTRL. After 24 h, the drugs were removed and after a washing step the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for 48 h. Afterwards, the cells were washed and finally treated with the third cytostatic drug (G, Ox or 5-FU) for additional 48 h. Cell confluence was monitored every 24 h. After a total therapy time of 120 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as Propidium iodide (for detection of dead cells) and Hoechst33342 (for detection of cell nuclei) staining. The statistical analysis was performed with the nuclei count data *via* Hoechst33342 staining. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil). Created with BioRender.com. 88

Figure 31 Long-term sequential therapy for 120 h using 3 cytostatic drugs without drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.25×10^4

parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or were left untreated as CTRL. After 24 h, the drugs were replaced with the second cytostatic drug (G, Ox or 5-FU). After 48 h, the cells were treated with the third cytostatic drug (G, Ox or 5-FU) for additional 48 h. After a total therapy time of 120 h the experiment was terminated with an endpoint analysis by Hoechst33342 (for detection of cell nuclei) staining for A) parental Panc1, Holo- and Paraclone cells as well as B) parental Panc89, Holo- and Paraclone cells. The graphs show the percentage of analyzed nuclei count normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, independent experiments n = 3. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil)..... 90

Figure 32 Illustration of long-term sequential therapy for 120 h with drug holiday. 0.25×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated with the first cytostatic drug (G, Ox or 5-FU) for 72 h or left untreated as CTRL. After 72 h, the drugs were removed and after a washing step fresh cell culture medium was added for 24 h drug holiday. After 24 h, medium was removed and the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 24 h. Cell confluence was monitored every 24 h. After a total therapy time of 120 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as Propidium iodide (for detection of dead cells) and Hoechst33342 (for detection of cell nuclei) staining. The statistical analysis was performed with the nuclei count data *via* Hoechst33342 staining. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil). Created with BioRender.com..... 91

Figure 33 Long-term sequential therapy for 120 h with drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.25×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 72 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 72 h, the drugs were replaced with fresh cell culture medium for 24 h drug holiday. After drug holiday, the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 24 h. After a total therapy time of 120 h, the experiment was terminated with an endpoint analysis by Hoechst33342 (for detection of cell nuclei) staining for A) parental Panc1, Holo- and Paraclone cells as well as B) parental Panc89, Holo- and Paraclone cells. The graphs show the percentage of analyzed nuclei count normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, independent experiments n = 5. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5-FU = 5-Fluorouracil)..... 92

Figure 34 Proposed strategies for optimization of PDAC treatment considering CSC and EMT phenotypes. (Ox = Oxaliplatin, G = Gemcitabine, 5-FU = 5-Fluorouracil, DH = drug holiday, CSC = cancer stem cell, EMT = Epithelial-to-Mesenchymal-Transition, Mono = monotherapy)..... 105

List of supplementary figures

- Supplementary figure 1 RNA sequencing-based subtyping of parental Panc1 and Panc89, Holo- and Paraclone cells. The subtyping analysis regarding B) classical-like and A, C) basal-like PDAC cell subtypes, based on the work of Moffit et al. ²⁸, revealed that Panc89 cell variants showed a strong tendency towards the basal-like subtype, but also classical-like characteristics. Panc1 cell variants showed no basal-like characteristics, but a tendency towards the classical subtype. Independent replicates n = 3. i
- Supplementary figure 2 Different cytokine release in cell culture supernatants of parental Panc1 and Panc89, Holo- and Paraclone cells. Cell culture supernatants from parental Panc1 and Panc89, Holo- and Paraclone cells were analyzed for the abundance of cytokines *via* Multiplex analysis *via* LEGENDplex™ Multi-Analyte Flow Assay Kit. The graphs show the cytokine concentrations of A) IL-8, B) IL-6, C) IL-10, and D) TNFα in cell supernatants normalized to the protein concentration. Statistical analysis was performed by Shapiro-Wilk normality test, followed by ordinary one-way ANOVA for parametric data and Kruskal-Wallis test for nonparametric data. Parametric data are represented as mean with SD and non-parametric data as median with interquartile range. Supernatants of n = 3 independent experiments were analyzed. Significance output $P \leq 0.033 = *$, $P \leq 0.002 = **$, $P \leq 0.001 = ***$. (Panc1 = Panc1 parental, Panc89 = Panc89 parental, Holo = Holoclone, Para = Paraclone).....ii
- Supplementary figure 3 Dose-Response-Analysis for parental Panc1 and Panc89, Holo- and Paraclone cells for Gemcitabine. 0.5×10^4 of parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates. After 24 h, the cells were treated with nine different concentrations of Gemcitabine or NaCl control. After 72 h, an endpoint analysis was performed by measuring the cell confluence and the number of dead cells *via* PI staining for A, C) parental Panc1, Holo- and Paraclone cells, as well as B, D) parental Panc89, Holo- and Paraclone cells. Statistical analysis was performed by [Inhibitor] vs. response Nonlin-fit in GraphPad Prism. The graphs present the percentage of cell confluence (upper panel) and of dead cells (lower panel) after 72 h treatment. Independent experiments n = 3 - 4. (PI = Propidium iodide) iii
- Supplementary figure 4 Dose-Response analysis (DRA) for parental Panc1 and Panc89, Holo- and Paraclone cells for Oxaliplatin. 0.5×10^4 of parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates. After 24 h, the cells were treated with nine different concentrations of Oxaliplatin or NaCl control. After 72 h, an endpoint analysis was performed by measuring the cell confluence and the number of dead cells *via* PI staining for A, C) parental Panc1, Holo- and Paraclone cells, as well as B, D) parental Panc89, Holo- and Paraclone cells. Statistical analysis was performed by [Inhibitor] vs. response Nonlin-fit in GraphPad Prism. The graphs present the percentage of cell confluence (upper panel) and of dead cells (lower panel) after 72 h treatment. Independent experiments n = 3 - 4. (PI = Propidium iodide) iv
- Supplementary figure 5 Dose-Response analysis (DRA) for parental Panc1 and Panc89, Holo- and Paraclone cells for 5-Fluorouracil (5-FU). 0.5×10^4 of parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates. After 24 h, the cells were treated with nine different concentrations of 5-FU or NaCl control. After 72 h, an endpoint analysis was performed by measuring the cell confluence and the number of dead cells *via* PI staining for A, C) parental Panc1, Holo- and Paraclone cells, as well as B, D) parental Panc89, Holo- and Paraclone cells. Statistical analysis was performed by [Inhibitor] vs. response Nonlin-fit in GraphPad Prism. The graphs present the percentage of cell confluence (upper panel) and of dead cells (lower panel) after 72 h treatment. Independent experiments n = 3 - 4. (PI = Propidium iodide, 5-FU = 5-Fluorouracil) v
- Supplementary figure 6 Short-term sequential therapy for 72 h with drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h,

the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs were replaced with fresh cell culture medium for 24 h drug holiday. After drug holiday, the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as cell staining with Propidium iodide (for detection of dead cells) for A, C) parental Panc1, Holo- and Paraclone cells as well as B, D) parental Panc89, Holo- and Paraclone cells. The graphs show the cell confluence and cell death (PI) normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, n = 4. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5 FU = 5 Fluorouracil) vi

Supplementary figure 7 Short-term sequential therapy for 72 h without drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs were replaced with the second cytostatic drug (G, Ox or 5-FU) for 48 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as cell staining with Propidium iodide (for detection of dead cells) for A, C) parental Panc1, Holo- and Paraclone cells as well as B, D) parental Panc89, Holo- and Paraclone cells. The graphs show the cell confluence and cell death normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, n = 3. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5 FU = 5 Fluorouracil) vii

Supplementary figure 8 Short-term sequential therapy for 72 h with 3 cytostatic drugs without drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.5×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs were replaced with the second cytostatic drug (G, Ox or 5-FU) for another 24 h. Finally, the cells were treated with the third cytostatic drug (G, Ox or 5-FU) for additional 24 h. After a total therapy time of 72 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as cell staining with Propidium iodide (for detection of dead cells) for A, C) parental Panc1, Holo- and Paraclone cells as well as B, D) parental Panc89, Holo- and Paraclone cells. The graphs show the cell confluence and cell death normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, n = 3. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5 FU = 5 Fluorouracil) viii

Supplementary figure 9 Long-term sequential therapy for 120 h with drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.25×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated for 72 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 72 h, the drugs were replaced with fresh cell culture medium for 24 h drug holiday. After drug holiday, the cells were treated with the second cytostatic drug (G, Ox or 5-FU) for additional 24 h. After a total therapy time of 120 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as cell staining with Propidium iodide (for detection of dead cells) for A, C) parental Panc1, Holo- and Paraclone cells as well as B, D) parental Panc89, Holo- and Paraclone cells. The graphs show the cell confluence and cell death normalized to untreated CTRL

(Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, n = 5. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5 FU = 5 Fluorouracil) ix

Supplementary figure 10 Long-term sequential therapy for 120 h in use of 3 cytostatic drugs without drug holiday revealed different treatment responses for parental Panc1 and Panc89, Holo- and Paraclone cells. 0.25×10^4 parental Panc1 and Panc89, Holo- and Paraclone cells were seeded in 96-well plates in duplicates. After 24 h, the cells were treated 24 h with the first cytostatic drug (G, Ox or 5-FU) or left untreated as CTRL. After 24 h, the drugs were replaced with the second cytostatic drug (G, Ox or 5-FU) for 48 h. Finally, the cells were treated with the third cytostatic drug (G, Ox or 5-FU) for additional 48 h. After a total therapy time of 120 h, the experiment was terminated with an endpoint analysis by measuring the cell confluence as well as cell staining with Propidium iodide (for detection of dead cells) for A, C) parental Panc1, Holo- and Paraclone cells as well as B, D) parental Panc89, Holo- and Paraclone cells. The graphs show the cell confluence and cell death normalized to untreated CTRL (Y-axis, scattered line) and the treatment scheme with the different cytostatic drugs (X-axis). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. Bars show data as mean with SEM and only significant comparisons with P-values ≤ 0.0001 are represented, n = 3. (CTRL = Control, G = Gemcitabine, Ox = Oxaliplatin, 5 FU = 5 Fluorouracil) x

List of tables

Table 1 Concentrations of the cytostatic drugs Gemcitabine, Oxaliplatin and 5-Fluorouracil used for IC50 determination <i>via</i> Dose-Response Analysis (independent experiments n = 3-4)	30
Table 2 IC50 values of Gemcitabine, Oxaliplatin, 5-Fluorouracil determined for Panc1 and Pan89 parental, Holo- and Paraclone cells by Dose-Response-Analysis (independent experiments n = 3-4).....	31
Table 3 Master mix composition for cDNA synthesis from RNA templates per sample	36
Table 4 LightCycler program for cDNA amplification in qPCR reaction	37
Table 5 Master mix composition for qPCR from cDNA templates per sample	37
Table 6 Heat-mediated antigen retrieval conditions for IHC analysis of mouse tissues	40
Table 7 Frequency Score (= % of positively stained cells) and Intensity score (intensity of staining) for IHC analysis of mouse tissues	40
Table 8 Determination of k_{Cell} confluence [%], k_{Nuclei} count [#] and respective k_{Mean} values for parental Panc1 and Panc89, Holo- and Paraclone cells (n = 3 independent experiments)	58

Erklärung

Hier mit versichere ich, Lisa-Marie Philipp (geboren am 07.04.1990 in Oldenburg i. H.), an Eides statt, dass ich die vorliegende wissenschaftliche Abhandlung mit dem Titel:

„The Impact of Clonal Heterogeneity and Plasticity on Malignancy Associated Properties and Therapy Response in Pancreatic Cancer“

nach den Regeln guter wissenschaftlicher Praxis eigenständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt habe. Dabei habe ich keine Hilfe, außer der wissenschaftlichen Beratung durch meine Doktormutter Prof. Dr. rer. nat. Susanne Sebens und durch die namentlich erwähnten Kooperationspartner in Anspruch genommen. Weder wurde diese Arbeit bisher im Rahmen eines Prüfungsverfahrens vorgelegt, noch wurde ein akademischer Grad entzogen. Teile dieser Arbeit sind bereits veröffentlicht und dementsprechend in dieser Arbeit als solche gekennzeichnet.



Kiel, Juni 2023

Lisa-Marie Philipp

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Kongresse, Fortbildungen

25.06.2020 - TopCare Prof. Marquardt "Update in molecular hepatocarcinogenesis: from sequential evolution to acquired resistance"

29.06.2020 - RTG: Prof. Kuemmerli "Siderophore-mediated social interactions in bacteria: from public goods games to treatments against pathogens"

30.06.2020 - Merck: Disease related Biomarkers in translational research studies

03.09.2020 - TopCare Prof. Dr. Tobias Maurer: "PSMA-based theranostics in prostate cancer"

15.09.2020 - Herr Prof. John Cryan, University College Cork, Ireland: "Gut Feelings- Translational approaches to Studying the Role of the Microbiota- Gut-Brain Axis in Modulating Brain & Behaviour".

Evolution by the Sea - Retreat 2021, **Posterpräsentation**

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Liver metastasis of pancreatic cancer: the hepatic microenvironment impacts differentiation and self-renewal capacity of pancreatic ductal epithelial cells

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is often diagnosed at advanced stages with the liver as the main site of metastases. The hepatic microenvironment has been shown to determine outgrowth of liver metastases. Cancer stem cells (CSCs) are essential for initiation and maintenance of tumors and acquisition of CSC-properties has been linked to Epithelial-Mesenchymal-Transition. Thus, this study aimed at elucidating whether and how the hepatic microenvironment impacts stemness and differentiation of disseminated pancreatic ductal epithelial cells (PDECs). Culture of premalignant H6c7-kras and malignant Panc1 PDECs together with hepatocytes and hepatic stellate cells (HSC) promoted self-renewal capacity of both PDEC lines. This was indicated by higher colony formation compared to cells cocultured with hepatocytes and hepatic myofibroblasts. Different Panc1 colony types derived from an HSC-enriched coculture were expanded and characterized revealing that holoclones exhibited an enhanced colony formation ability, elevated and exclusive expression of the CSC-marker Nestin and a more pronounced mesenchymal phenotype compared to paraclones. Moreover, Panc1 holoclone cells showed an increased tumorigenic potential *in vivo* leading to formation of undifferentiated tumors in 7/10 animals, while inoculation of paraclone cells only led to formation of tumors in 2/10 animals being smaller in number and size. Holoclone tumors were characterized by elevated expression of mesenchymal markers, complete loss of E-cadherin expression and high

expression of Nestin. Finally, Etanercept-mediated TNF- α blocking partly reversed the mesenchymal CSC-phenotype of Panc1 holoclone cells. Overall, these data provide evidence that the hepatic microenvironment determines stemness and differentiation of PDECs, thereby substantially contributing to liver metastases of PDAC.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC), the most common pancreatic neoplasia, is characterized by its particularly poor prognosis with a 5-year survival rate of only around 8% [1] and an ongoing increase in death rate [2]. Due to the lack of early and specific symptoms, the majority of patients presents with advanced or even metastasized disease which only leaves palliative treatment. The only curative option that is eligible to about 20% of patients is surgery and even after surgery, the 5-year survival is poor owing to recurring or metastatic tumor growth [3, 4].

The early occurrence of distant metastases even after removal of the primary tumor supports the idea of epithelial/tumor cell dissemination as an early event during malignant progression [5]. As a prerequisite for dissemination, epithelial/tumor cells undergo Epithelial-Mesenchymal-Transition (EMT) leading to the acquisition of a more motile and invasive phenotype. This is associated with reduced expression of epithelial proteins like E-cadherin and upregulation of mesenchymal markers such as N-cadherin, Vimentin, L1CAM or the transcription factor Zeb1 [6–8]. Furthermore, loss of cell differentiation may coincide with the acquisition of cancer stem cell (CSC)-characteristics of the tumor cells [9–11]. Mani *et al.* [9] were the first who demonstrated that breast cancer cells that have undergone EMT acquire a stem cell-like phenotype, and likewise, stem cell-like cells resemble cells that have undergone EMT.

CSCs are a small group of cancer cells within a cancer cell population with the unique ability to self-renew and to generate more differentiated cells. Thus, CSCs have been shown to be essential for tumor initiation, development, progression, recurrence and therapy resistance in various cancer entities, including PDAC [12–14]. Recent studies indicate that CSC-properties can be gained and lost depending on the microenvironment [11, 15–18], indicating that CSCs are not a stable cell population. Several markers, e.g. ABCG2, CD133, CD24 and CD44, have been proposed for CSCs in PDAC indicating a high heterogeneity and plasticity within the CSC population [19–21]. Besides the above mentioned markers, the transcription factor Nanog and the intermediate filament Nestin seem to play a role in the maintenance of CSC-properties [22, 23]. Moreover, it has been shown that Nestin impacts cell motility and EMT-properties in PDAC cell lines. Knockdown of Nestin in PDAC cells led to a reduced tumor incidence and volume as well as reduced formation of metastases in a murine PDAC model [24, 25]. One of the main

metastatic sites of PDAC next to lung and peritoneum is the liver. While an impact of the liver microenvironment on EMT of prostate and breast cancer cells has been shown [26, 27], only few studies have addressed the role of the liver microenvironment on the development of PDAC metastases. Next to hepatocytes, hepatic stellate cells (HSCs) and their activated inflammatory counterpart, the hepatic myofibroblasts (HMFs), play an important role in liver tissue homeostasis, especially during injury and inflammation [28–31]. Furthermore, HMFs are also thought to be important for establishing a prometastatic microenvironment [29, 32]. A previous study of our group has provided evidence that HSCs contribute to induction and maintenance of a dormant phenotype in pancreatic ductal epithelial cells (PDECs) of different malignant potential. In contrast, HMFs have been shown to support the reversal of this dormant stage and foster metastatic outgrowth [33].

However, until now it is unclear how the hepatic microenvironment impacts on disseminated pancreatic ductal epithelial cells regarding their EMT- and CSC-characteristics. Thus, the present study aimed at analyzing the influence of different hepatic stromal conditions, displayed by hepatocytes and hepatic stellate cells or myofibroblasts, respectively, on cell growth, differentiation and self-renewal of PDECs by using an indirect coculture approach *in vitro* and a tumorigenicity PDAC mouse model *in vivo*.

RESULTS

The hepatic microenvironment supports self-renewal of premalignant and malignant PDECs

When disseminated PDECs settle in a secondary organ, they are exposed to a new microenvironment which impacts their growth behavior and survival [33]. In the liver, hepatocytes as well as hepatic stromal cells like HSCs and, upon inflammatory processes, HMFs play an important role in this scenario. Since it is still poorly understood how the hepatic microenvironment impacts CSC-features and differentiation of PDECs, we employed an indirect coculture system with different hepatic stromal cells and H6c7-*kras* cells (harboring only mutant *kras* as a PDAC-associated mutation) or malignant Panc1 cells, the latter exhibiting several PDAC-associated genetic and epigenetic alterations.

In order to mimic a physiological microenvironment, coculture of either Panc1 or H6c7-*kras* cells was performed with hepatocytes alone (co H) or hepatocytes together with 5% HSC (co H+5HSC). For mimicking the

inflamed liver micromilieu, Panc1 or H6c7-kras cells were cocultured with hepatocytes in the presence of 5% HMF (co H+5HMF). To study the influence of the different hepatic stromal conditions on self-renewal capacity of PDECs, colony formation assays (CFAs) were performed with Panc1 and H6c7-kras cells after 6 days of mono- or coculture. As expected, the ability to form colonies was higher in malignant Panc1 cells than in premalignant H6c7-kras cells independent of the culture conditions (Figure 1A). In both PDEC lines, colony formation was most pronounced after HSC-enriched coculture and lowest after coculture with hepatocytes and HMF (Figure 1A). Panc1 cells mostly formed paraclones which are supposed to be comprised of more differentiated cells, but they also gave rise to a considerable amount of mero- (25.0–38.8%) and holoclones (2.5–5.9%), the latter being supposed to contain the highest proportion of CSCs (Figure 1B). Thus, while the number of colonies of Panc1 cells was highest under HSC-enriched conditions, the formation of distinct colony types was not affected by the different hepatic stromal conditions. H6c7-kras cells also predominantly formed paraclones but in contrast to Panc1 cells, rarely formed meroclones (2.8–8.4%) or holoclones (0.0–0.6%) (Figure 1B). To confirm these findings, coculture experiments with human hepatocytes, hepatic stellate cells and hepatic myofibroblasts and either H6c7-kras, Panc1 cells or another PDAC cell line Panc89 were performed. In accordance with our findings described in Figure 1, malignant Panc1 and Panc89 cells formed more colonies than H6c7-kras cells and colony formation was highest in all three PDEC lines under coculture with human hepatocytes and 5% human hepatic stellate cells (Supplementary Figure 1). Overall, these data suggest that the self-renewal capacity of PDECs is maintained in the liver microenvironment even in an HSC enriched liver microenvironment resembling a physiological liver.

Panc1 holoclones display enhanced formation of holoclones and Nestin expression compared to paraclones

To characterize pancreatic CSCs that are able to survive and proliferate in HSC-enriched coculture conditions, a single cell dilution assay was performed with all three PDEC lines after coculture with H+5HSC and also after monoculture (Figure 2A). In parallel to the expression of different clone types, CFAs were regularly performed to confirm colony types and only cells with a robust phenotype were considered for further *in vitro* and *in vivo* experiments (Figure 2, Supplementary Figure 2). Clonal dilution and expansion of malignant Panc1 (Figure 2B, 2C) and Panc89 cells (Supplementary Figure 2) led to stable generation of para- and holoclones while in premalignant H6c7-kras cells only para- and meroclones could be stably established (Supplementary Figure 2). The total number of formed colonies was

similar for both colony types obtained with Panc1 cells (Figure 2B). Moreover, Panc1 paraclone cells predominantly formed paraclones again and only rarely mero- and holoclones (Figure 2C). In contrast, H6c7-kras meroclones and Panc89 holoclones formed more colonies than the respective paraclone cells (Supplementary Figure 2A). H6c7-kras paraclone cells predominantly formed paraclones and only rarely mero- and holoclones while H6c7-kras meroclones also predominantly formed paraclones but concomitantly 32% meroclones (Supplementary Figure 2B). Panc89 holoclone cells predominantly formed holo- and meroclones (45.0% and 51.1%) along with 17% paraclones while paraclone cells formed mostly meroclones (62.9%) and lower numbers of holo- (21.1%) and paraclones (15.9%) (Supplementary Figure 2B). The latter observation might be explained by the fact that most isolated paraclones stopped proliferation during expansion and only those clones could be expanded containing a higher amount of holo- and meroclone cells. Having verified the differences in self-renewal capacity, cells from holo-/mero- and paraclones were further characterized with respect to CSC-marker expression. Holoclones from malignant PDECs showed elevated expression of Nestin, Nanog and ABCG2 of which Nestin was most strikingly elevated (Figure 2D, Supplementary Figure 2C). Premalignant H6c7-kras cells hardly forming any holoclones showed only significantly elevated Nanog expression (Supplementary Figure 2C). To verify these expression differences of the two CSC-markers on the protein level, immunofluorescence stainings were performed in Panc1 cells revealing considerable Nanog expression in both clone types, but with a higher number of strongly stained cells in paraclones (Figure 2E, 2F). In contrast, Nestin was exclusively expressed by holoclone-derived Panc1 cells (Figure 2E, 2F). Overall, these data demonstrate that premalignant H6c7-kras cells showed less pronounced self-renewal capacity indicated by the absence of established holoclone cells along with the absence of Nestin expression but pronounced Nanog expression. In contrast, holoclone cells of malignant Panc1 and Panc89 cells were characterized by pronounced self-renewal capacity and elevated Nestin expression.

Panc1 holoclone cells derived from an HSC-enriched coculture exhibit an increased tumorigenic potential leading to formation of undifferentiated (mesenchymal) tumors

Next, it was investigated whether elevated self-renewal capacity of holoclones expanded from HSC-enriched coculture is associated with an enhanced tumorigenic potential *in vivo*. Since the phenotype of expanded Panc1 holo- and paraclone cells was most stable, 10⁴ holo- or paraclones-derived Panc1 cells were intrasplenically inoculated in SCID-beige mice and tumor formation was monitored by ultrasound over a period of

almost 5 months (Figure 3A). While inoculation of 10^4 Panc1 paraclone cells only led to macroscopic tumor formation in pancreas and lung in 1/10 animals each, Panc1 holoclone cells led to pancreatic tumor growth in 7/10

animals and formation of macroscopic liver metastases in 3/10 animals (Figure 3B). Detection of inoculated Panc1 cells by immunofluorescence staining targeting human cytokeratin revealed additional microscopic tumoral

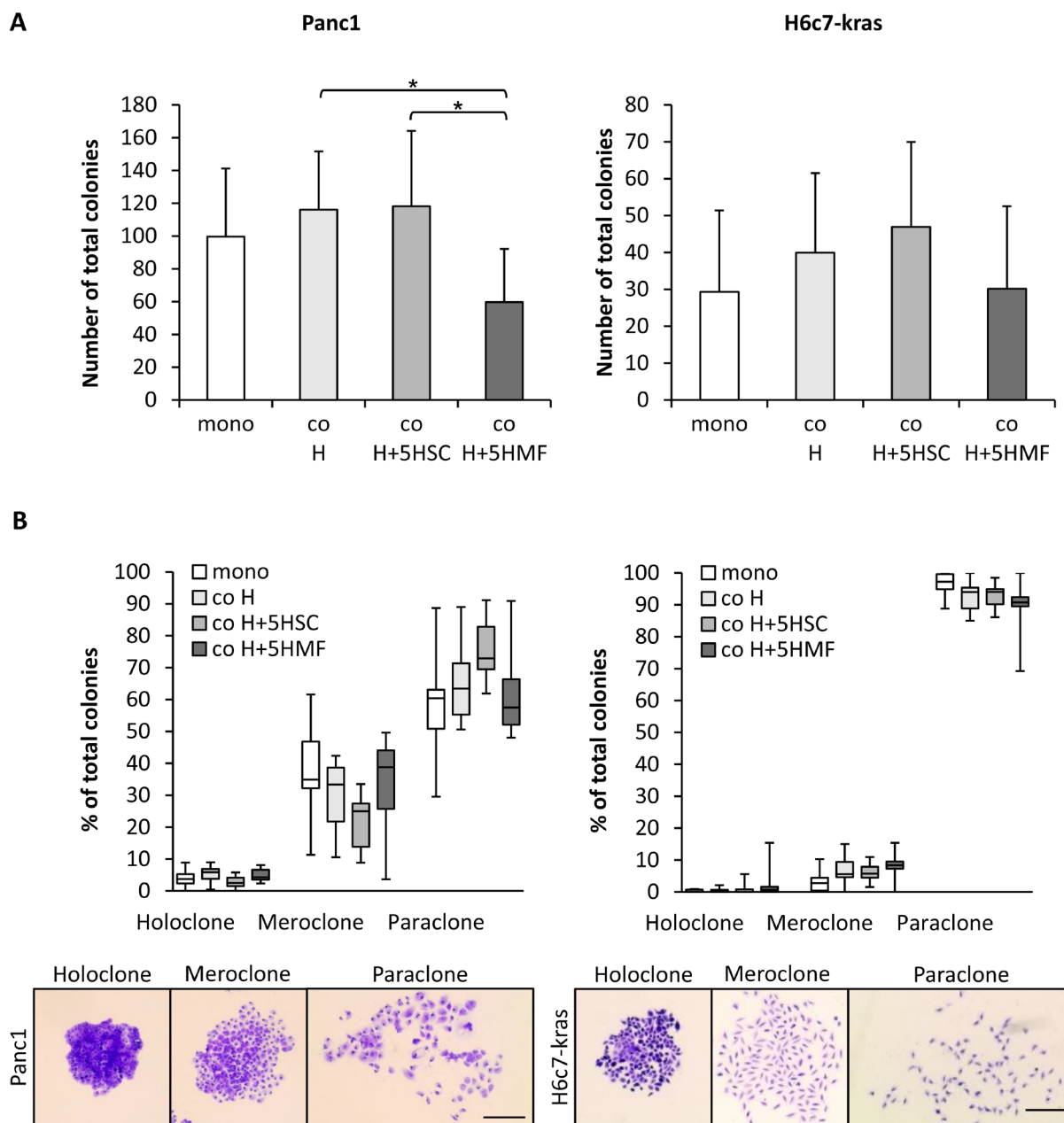


Figure 1: The hepatic microenvironment supports self-renewal of PDECs. Panc1 and H6c7-kras cells were either monocultured (mono) or indirectly cocultured in different experimental hepatic environments, consisting of hepatocytes alone (co H) or hepatocytes enriched with 5% HSC (co H+5HSC) or 5% HMF (co H+5HMF), respectively, for 6 days. (A, B) After 6 day culture under the described conditions, PDECs were detached and 400 cells seeded for colony formation which was assessed after crystal violet staining on day 10. Only colonies containing more than 50 cells were counted and (A) the total number of colonies and (B) the proportion of different colony types of total number of colonies were determined. Due to reasons of clarity, significant differences are not marked in this chart. Data are presented as mean and standard deviation or median and quartiles (Q1 as 25% and Q3 as 75%) of 6 to 7 independent experiments. Below, representative images of crystal violet-stained holo-, mero- and paraclones of Panc1 and H6c7-kras cells are shown. Scale bar 250 μ m. * indicates statistically significant differences ($p \leq 0.05$).

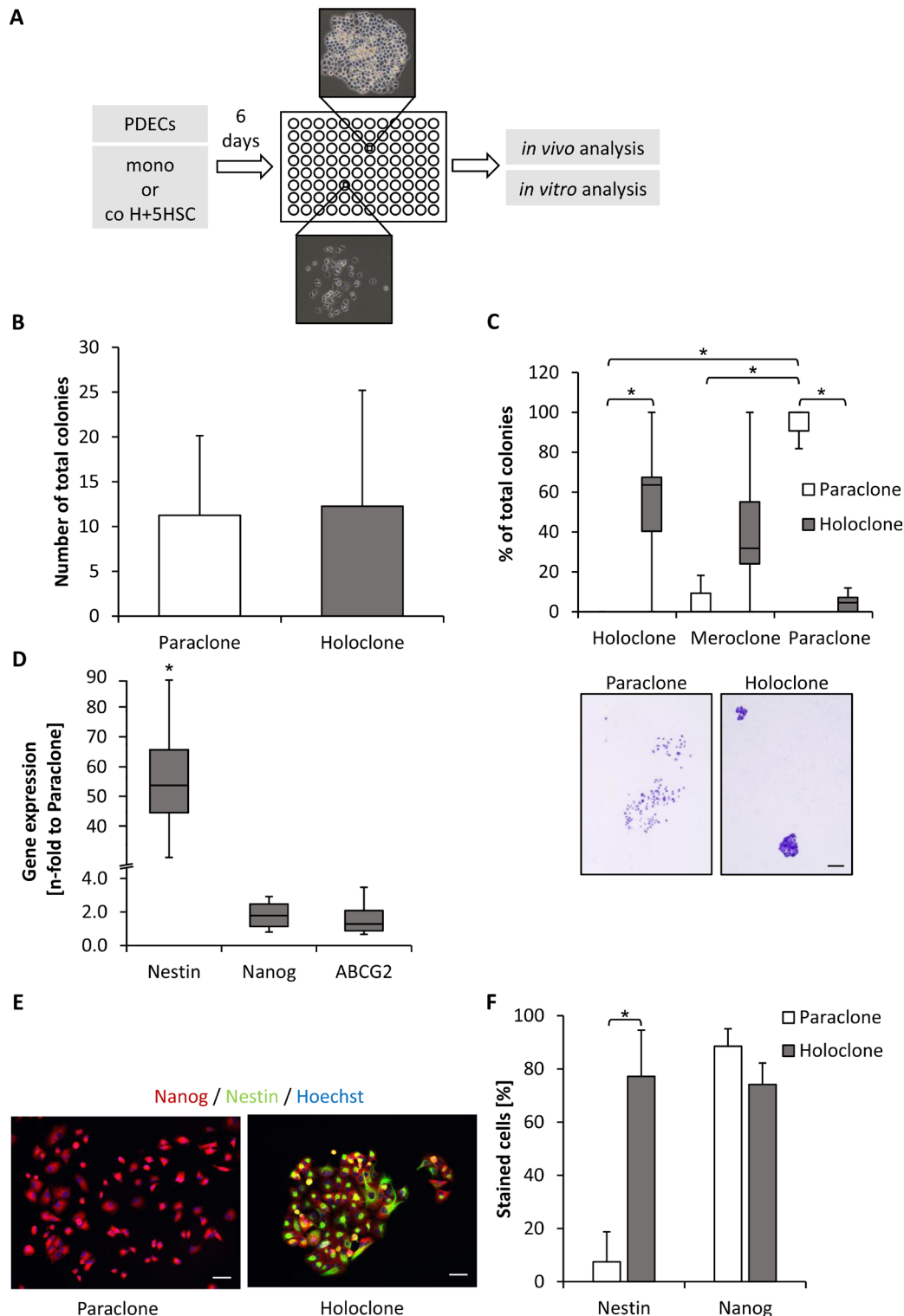


Figure 2: Panc1 holoclones display enhanced formation of holoclones and Nestin expression compared to paraclones. (A) Scheme of generation and expansion procedure of different PDEC clones. After 6 day culture as depicted, single PDECs were seeded in a 96-well plate until single colonies were visible and their phenotype was assessed and confirmed by CFA. Only cells with stable phenotype were considered for further expansion and experiments. (B, C) In order to assess self-renewal capacity of expanded Panc1 holo- and paraclone cells, 400 cells were seeded for colony formation which was assessed after crystal violet staining on day 10. Only colonies containing more than 50 cells were counted and (B) the total number of colonies and (C) the proportion of different colony types of total

number of colonies was determined. Data are presented as mean and standard deviation or median and quartiles (Q1 as 25% and Q3 as 75%) of 6 to 7 independent experiments. Below, representative images of crystal violet-stained Panc1 para- and holoclones are shown. Scale bar 250 μ m. (D) RT-qPCR analysis of Nestin, Nanog and ABCG2 mRNA-expression normalized to GAPDH as housekeeper in Panc1 para- and holoclones. Data are presented as n-fold gene expression of paraclones and as median and quartiles (Q1 as 25% and Q3 as 75%) of 4 independent experiments. (E, F) Immunofluorescence staining of CSC-markers Nanog (red) and Nestin (green) in Panc1 paraclone and holoclone cells after *in vitro* expansion. Hoechst staining (blue) was performed to mark nuclei. Scale bar 25 μ m. (F) Percentage of positively stained cells was determined by considering all cells contacting a horizontal line in 5 randomly chosen fields of view. Proportion of stained cells to total cell number is shown. Data are presented as mean and standard deviation of 3 independent experiments. * indicates statistically significant differences ($p \leq 0.05$).

lesions in both holoclone- and paraclone-inoculated animals. However, more and larger microscopic lesions were detected in pancreas, liver and lung of animals inoculated with holoclone-derived cells (Figure 3C).

In line with the *in vitro* data, holoclone-derived tumoral lesions exhibited strong expression of Nestin. Moreover, only the single paraclone-derived pancreatic tumor, but not the corresponding small hepatic tumoral lesion showed weak Nestin expression (Figure 4A).

Furthermore, both pancreatic and hepatic holoclone tumors displayed moderate to strong expression of the mesenchymal markers Vimentin, N-cadherin, Integrin alpha V as well as CSC-marker CD44 as detected in immunohistochemical stainings. In contrast, expression of epithelial E-cadherin was completely absent in tumor cells and only found in adjacent non-tumoral pancreatic or liver epithelial cells (Figure 4B). Due to the small size of the only hepatic lesion derived from paraclones, we could not determine EMT-marker expression in this tissue. However, in the paraclone-derived pancreatic tumor similar expression patterns of Vimentin, N-cadherin, Integrin alpha V, CD44 and E-cadherin could be detected as in holoclone tumors (Figure 4B). These *in vivo* data suggest that Panc1 holoclone cells derived from an HSC-enriched coculture exhibit an increased tumorigenic potential leading to formation of undifferentiated (mesenchymal) tumors predominantly in pancreas and liver.

Panc1 holoclone cells exhibit a more pronounced mesenchymal phenotype than paraclones which is partly dependent on TNF- α

Since holoclone-derived tumors exhibited characteristics of EMT, Panc1 holo- and paraclone cells were characterized with respect to further molecular aspects of EMT. In line with our *in vivo* data, Panc1 holoclone cells exhibited a reduced E-cadherin expression and higher expression levels of L1CAM, Vimentin, Zeb1 and Tumor Necrosis Factor-alpha (TNF- α) compared to paraclones indicating a stronger mesenchymal phenotype (Figure 5A). Western blot analyses confirmed elevated L1CAM and Zeb1 expression in holoclones (Figure 5B). Moreover, a higher expression of the activated (phosphorylated) p65 subunit of NF- κ B could be detected in Panc1 holoclone cells fitting together with the elevated TNF- α expression in these cells (Figure 5A, 5B). Due to low abundance of E-cadherin-expressing Panc1 cells,

immunofluorescence staining was performed instead of western blot analysis revealing single E-cadherin positive cells within the paraclone cell population (Figure 5C). In contrast, no E-cadherin expressing cells were visible in holoclone-derived Panc1 cells (Figure 5C), further supporting the more pronounced mesenchymal phenotype of these cells. EMT profiling of H6c7-kras mero- and paraclone cells revealed that H6c7-kras meroclone cells exhibited considerable expression of mesenchymal proteins as observed in Panc1 holoclone cells. However, no reduction in E-cadherin expression could be detected indicating a mixed epithelial-/mesenchymal phenotype of H6c7-kras meroclone cells (Supplementary Figure 3A). In contrast, Panc89 holoclone cells showed significant reduction in E-cadherin expression but concomitantly either reduction or no difference in the expression of mesenchymal proteins compared to the paraclone cells (Supplementary Figure 3B) pointing to a mixed epithelial-/mesenchymal CSC-like phenotype. Overall, these findings indicate that acquisition of CSC-properties and an EMT phenotype occurs stepwise in PDECs and that premalignant and malignant PDECs are heterogeneous with regard to EMT/CSC-properties. This also implies that PDECs with a high CSC-potential can differ with respect to their differentiation.

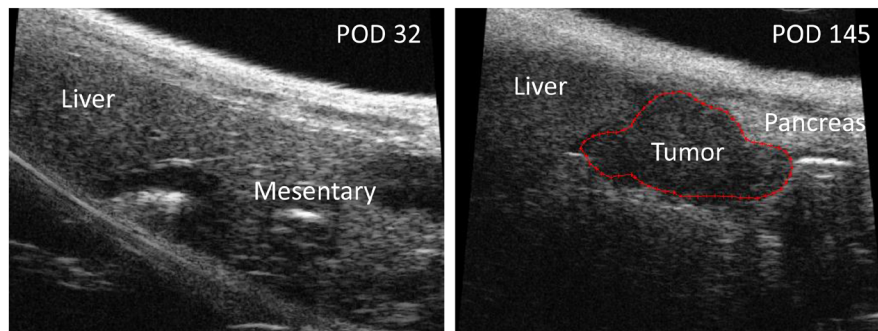
Since mesenchymal Panc1 holoclones (and also H6c7-kras meroclones) exhibited higher TNF- α expression, we next investigated whether this expression change might contribute to the maintenance of the more pronounced CSC-phenotype of holoclone-derived Panc1 cells. Therefore, Panc1 holoclone cells were treated either with Etanercept for 72 h to block TNF- α activity or cultured under comparable conditions in the absence of Etanercept as control. Blockade of TNF- α did not influence expression of E-cadherin, but reduced expression of the mesenchymal proteins L1CAM, Vimentin and Zeb1. In addition, expression of CSC-markers Nestin and predominantly Nanog was also reduced (Figure 5D). Although TNF- α blockade did not diminish the total number of formed colonies (Figure 5E), it considerably reduced formation of CSC-enriched holoclones in favor of paraclone formation, being consistent with the reduced CSC-marker expression (Figure 5F). These data suggest that the mesenchymal phenotype and CSC-properties of Panc1 holoclone cells are sustained by TNF- α indicating that this inflammatory cytokine is a common mediator of EMT and cancer stemness in mesenchymal PDAC cells.

DISCUSSION

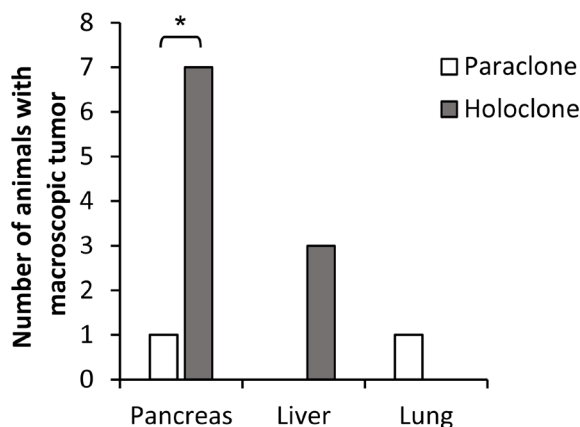
We have recently shown that the hepatic microenvironment represents an important determinant in induction and reversal of dormancy during PDAC metastases formation and that inflammatory alterations of the liver drive the outgrowth of PDAC metastases growth [33]. Importantly, self-renewal capacity as well as differentiation potential, both being sustained by CSCs, are mandatory for initiation and progression of tumors [13, 14, 34]. Therefore, this study aimed at extending the above mentioned findings by gaining a better understanding of the mechanisms by which the liver microenvironment impacts cancer stemness in PDAC metastases. For this purpose, we modeled the presence of disseminated PDECs as well as their heterogeneity in the hepatic microenvironment *in vitro* by coculturing H6c7-kras (epithelial-mesenchymal phenotype, harboring only kras

mutation), Panc89 cells (moderately differentiated/ epithelial-mesenchymal phenotype, harboring several PDAC associated genetic alterations) and Panc1 cells (poorly differentiated/ mesenchymal phenotype, harboring several PDAC associated genetic alterations) either alone, in the presence of hepatocytes alone or together with 5% HSC (mimicking physiological conditions) or 5% HMF (mimicking inflamed conditions). As expected, colony formation of malignant Panc89 and Panc1 cells was more pronounced compared to H6c7-kras cells with incomplete malignant transformation. However, colony formation ability of all three PDEC lines was most pronounced in an HSC-enriched liver microenvironment which suggests that CSC-properties are well maintained and promoted by a physiological liver micromilieu. The observation that all PDEC lines formed fewer colonies after coculture under inflamed liver conditions (H+5% HMF) might be explained by the fact that HMFs promote PDEC proliferation [33]

A



B



C

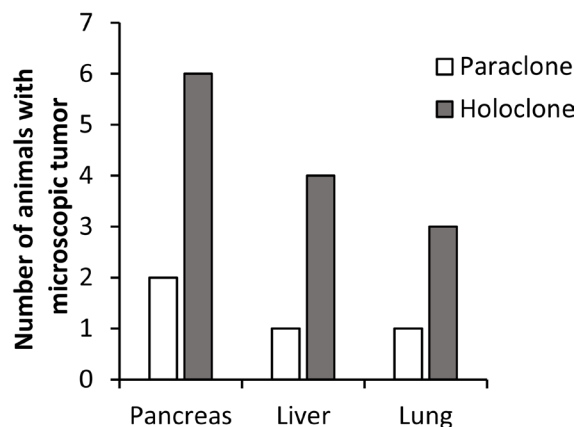
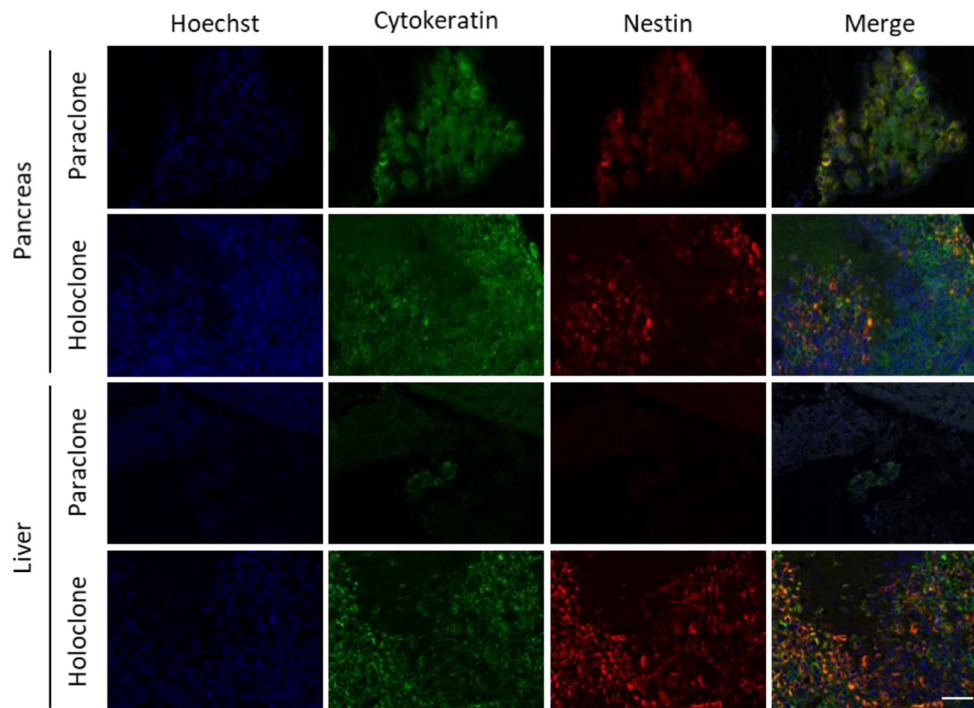


Figure 3: Panc1 holoclone cells exhibit an increased tumorigenic potential *in vivo*. Tumorigenic potential of Panc1 holoclone and paraclone cells derived from an HSC-enriched coculture was investigated by intrasplenic inoculation of 10^4 holoclone or paraclone cells into SCID-beige mice ($n = 10$ animals/cell type). (A) Representative sonographic images of an animal inoculated with Panc1 holoclone cells on day 32 and 145 post operation (POD). A macroscopic tumor lesion is encircled by red line (right panel). (B) Number of animals with macroscopic tumors in pancreas, liver and lung and (C) microscopic tumors in pancreas, liver and lung detected by immunofluorescence staining of human cytokeratin in formalin-fixed paraffin-embedded tissue sections of resected organs. * indicates statistically significant differences ($p \leq 0.05$).

A



B

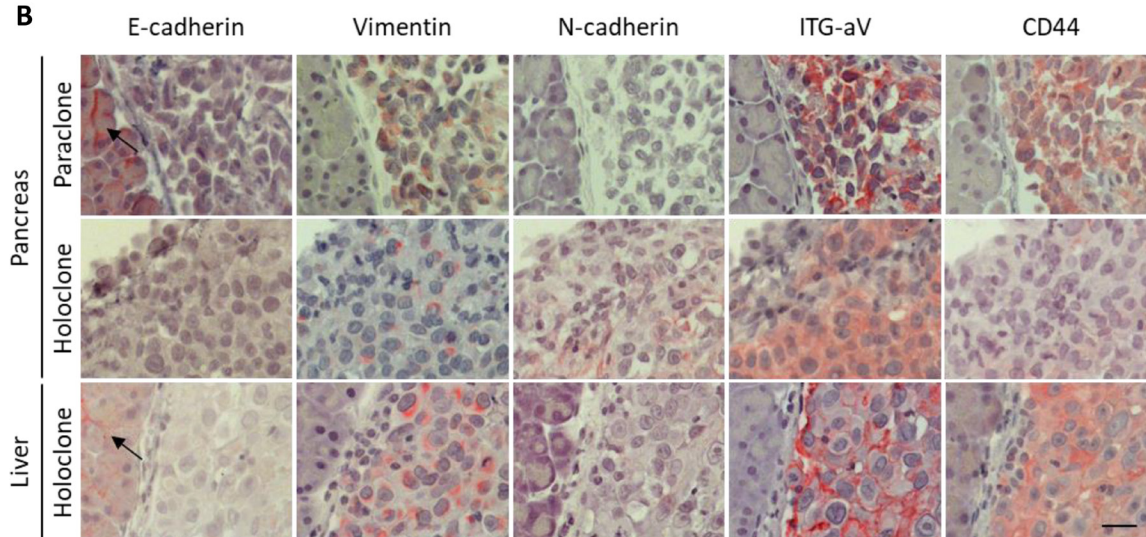


Figure 4: Panc1 holoclone tumors exhibit a pronounced mesenchymal phenotype along with elevated Nestin expression. Tumorigenic potential of Panc1 holoclone and paraclone cells derived from an HSC-enriched coculture was investigated by intrasplenic inoculation of 10^4 holoclone or paraclone cells into SCID-beige mice ($n = 10$ animals/cell type). Formalin-fixed paraffin-embedded tissue sections of pancreatic and corresponding liver sections were (A) immunofluorescently co-stained for human Cytokeratin (green) for detection of human Panc1 cells and the CSC-marker Nestin (red). Hoechst staining was performed to mark nuclei (blue). Scale bar 50 μ m. (B) Pancreatic and hepatic tissues were immunohistochemically stained for E-cadherin, Vimentin, N-cadherin, Integrin alpha V (ITG-aV) and CD44. Arrows indicate E-cadherin-expressing non-neoplastic tissue cells. Haemalum staining was performed to counterstain nuclei. Scale bar 25 μ m.

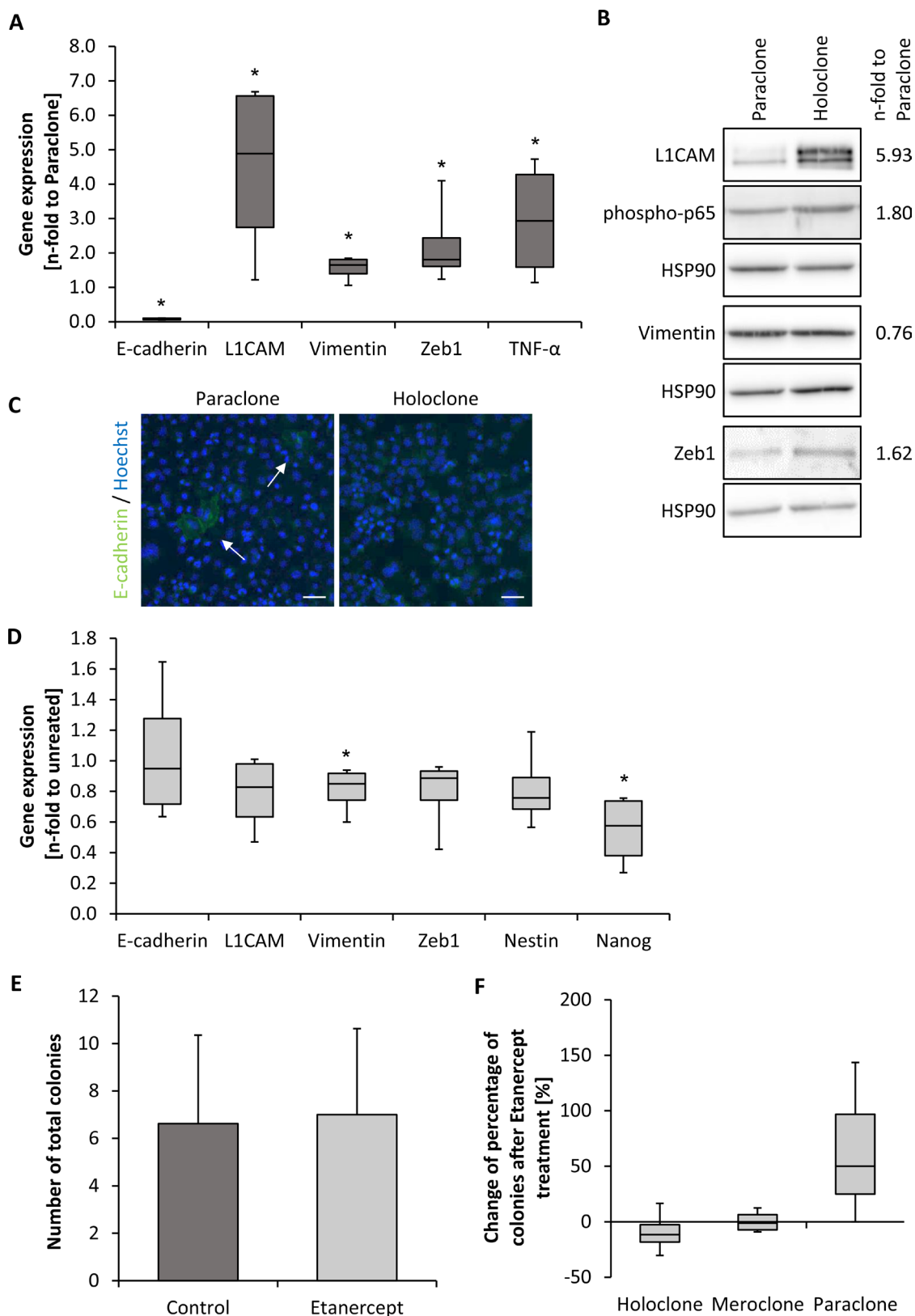


Figure 5: Panc1 holoclone cells exhibit a pronounced mesenchymal phenotype which is partly dependent on TNF- α signaling. EMT-profile of Panc1 holo- and paraclone cells derived from expansion procedure. (A) RT-qPCR analysis for expression of E-cadherin, L1CAM, Vimentin, Zeb1 and TNF- α normalized to the housekeeping gene GAPDH and presented as n-fold expression compared to expression in paraclone cells. (B) Western blot analysis of L1CAM, Vimentin, phospho-p65 and Zeb1 in Panc1 para- and

holoclone cells. HSP90 was used as loading control. One representative result of 4 independent experiments is shown. Numbers at the right side of the bands indicate average band intensities of holoclone probes determined by densitometric analysis. Values of the proteins of interest were divided by the values of the corresponding loading control (HSP90) and normalized to values of paraclones. (C) Immunofluorescence staining of E-cadherin (green) in Panc1 para- and holoclone cells. Hoechst staining (blue) was used for nuclei detection. Arrows indicate E-cadherin-expressing cells. Scale bar 50 μ m. (D) RT-qPCR analysis for expression of E-cadherin, L1CAM, Vimentin, Zeb1 as well as Nestin and Nanog in Panc1 holoclone cells that were left untreated or treated with 10 μ g/ml Etanercept for 72 hours. Specific gene expression was normalized to the housekeeping gene GAPDH and presented as n-fold expression compared to control. (E, F) For examining the influence of Etanercept on colony formation, 400 Panc1 holoclone cells were seeded in duplicates and treated with 10 μ g/ml Etanercept on day 1 and 7 or left untreated as control. Colony formation was assessed after crystal violet staining on day 11. Only colonies containing more than 50 cells were counted and (E) the total number of colonies was determined as well as (F) the proportion of different colony types of total number of colonies. Data are shown as percentage change of Etanercept-treatment compared to control. Data are presented as mean and standard deviation or median and quartiles (Q1 as 25% and Q3 as 75%) of 4 independent experiments. * indicates statistically significant differences ($p \leq 0.05$).

leading to dilution of the CSC pool. To further characterize the clone types formed under HSC-enriched conditions, holo-/meroclones and paraclones derived from single cocultured cells were expanded and analyzed. For PDAC, many CSC-markers have been proposed including Nestin and Nanog [22–24]. In contrast to a study by Skoda *et al.* [35], in which Nestin was expressed in 95% of all cells derived from primary PDAC cell lines, pointing to a common expression of this protein in the entire tumor cell population, we found Nestin to be differentially expressed in Panc1 and Panc89 para- and holoclones with Nestin being exclusively expressed in holoclone cells. Supporting the view that Nestin expression is a hallmark of cells with the highest self-renewal ability, meroclones formed by premalignant H6c7-kras cells hardly displayed any Nestin expression. Moreover, an increased Nestin expression correlated with enhanced formation of holoclones of Panc1 cells *in vitro* as well as an enhanced tumorigenicity *in vivo* leading to formation of Nestin-positive tumors in SCID-beige mice. These findings are consistent with other studies reporting that Nestin inhibition led to reduced cell migration and invasion *in vitro* as well as diminished metastases formation *in vivo* [12, 24]. In contrast to studies in prostate cancer, in which only inoculation of holoclones or meroclones resulted in tumor growth [36, 37], injection of Panc1 paraclone cells also led to tumor formation albeit in fewer animals (7/10 for holoclones, 2/10 for paraclones) as well as smaller in number and size compared to tumors formed by holoclone cells. Important to note, either clone cell population - even though expanded from a distinct colony type - was heterogeneous so that holoclone cells also formed paraclones and *vice versa* paraclones also contained single holoclone cells (see Figure 2B). Thus, formation of tumors by paraclone cells might be explained by the fact that holoclone cells, being present in the inoculated tumor cell population, led to scattered tumor formation. Alternatively, a certain number of disseminated paraclone cells may have regained CSC-/holoclone-properties owing to the influence of the microenvironment. This might also explain the Nestin expression detected in the single pancreatic paraclone lesion and, again, suggests that either the lesion originated from one of the few Nestin-positive holoclone cells within the inoculated paraclone cell

population or Nestin expression was induced once the paraclone cells had settled in the pancreas. Interestingly, Nestin expression was not found in the hepatic paraclone lesion supporting the role of the microenvironment as a determining factor of cancer stemness and tumor formation [31, 38]. It has been shown that the transition between CSC- and non-CSC-states is linked to EMT and that its reversal, the Mesenchymal-Epithelial-Transition (MET), in cancer cells is highly dependent on soluble factors that e.g. might be released by the local environment [9, 10, 26]. Accordingly, Panc1 holoclone cells did not only exhibit high levels of Nestin expression, but also showed a pronounced mesenchymal phenotype associated with reduced expression of E-cadherin as well as increased expression of mesenchymal markers *in vivo* and *in vitro* indicating a mesenchymal phenotype with pronounced CSC-potential. H6c7-kras meroclone cells also exhibited enhanced expression of mesenchymal proteins compared to paraclones but concomitantly displayed E-cadherin expression pointing to a mixed epithelial-/mesenchymal phenotype along with limited CSC-potential. Finally, Panc89 holoclone cells showed marked formation of holoclones along with elevated Nestin expression compared to paraclone cells and exhibited reduced E-cadherin expression but no increased expression of mesenchymal proteins indicating a mixed epithelial-/mesenchymal phenotype but with pronounced CSC-potential. Overall, these data support the view that PDAC cells with a high CSC-potential (formation of holoclones *in vitro* and high tumorigenicity *in vivo*) do not necessarily have to exhibit a full mesenchymal phenotype (reduced expression of epithelial proteins and enhanced expression of mesenchymal proteins) in order to initiate tumor/metastases formation. It can be speculated that downregulation of E-cadherin is an essential step in increasing Nestin expression thereby conferring self-renewal capacity in PDAC cells as it has been shown in lung adenocarcinoma cells [39]. Known factors that promote EMT and CSC-properties are TNF- α or Transforming Growth Factor-beta1 (TGF- β 1) [9, 10, 23, 40]. Indeed, the mesenchymal phenotype along with the CSC-characteristics in Panc1 holoclone cells could be partly reduced by blocking TNF- α , resulting in the formation of higher numbers of paraclones at the expense of holoclone abundance. These results further

support the view of an interconnection between EMT-characteristics and CSC-potential in PDAC cells. In line with these findings, it could be shown that Nestin overexpression increases cell motility along with a more mesenchymal phenotype *in vitro*, whereas its downregulation favored an epithelial phenotype and reduced tumor incidence and size [12, 23, 41]. Owing to the fact that blockade of TNF- α did not completely inhibit colony formation (and with this self-renewal) of Panc1 cells points to other factors being involved in the regulation of differentiation and stemness properties in these cells. In this context it might be of interest that Panc1 cells secrete elevated levels of TGF- β 1 [42, 43]. Since TGF- β 1 expression did not significantly differ between holoclone and paraclone Panc1 cells, we did not further investigate its role in regulating holoclone and paraclone behavior of these cells. Krebs *et al.* [44] have recently identified Zeb1 as a major factor for the initiation of metastatic growth in pancreatic cancer with its absence being connected to reduced stemness and cellular plasticity [44, 45]. Enhanced expression of Zeb1 has also been associated with an inflamed microenvironment [46]. De Cock *et al.* [46] observed the outgrowth of lung metastasis from previously seeded latent disseminated tumor cells after induction of inflammation. This inflammatory-driven metastatic outgrowth was consistent with a causal activation of EMT being mediated by Zeb1 and Twist. In accordance with these findings, Zeb1 expression was significantly higher in Panc1 holoclone cells compared to paraclone cells and was also negatively affected by TNF- α blockade. Thus, these data also support a benefit for TNF- α blockade as therapeutic strategy for prevention or inhibition of PDAC primary and metastatic tumor growth. Egberts *et al.* [47] have already shown that TNF- α increases tumor growth and invasiveness of PDAC cells and that inhibition of TNF- α with Infliximab or Etanercept significantly reduces tumor burden in PDAC xenograft models. Moreover, TNF- α blockade performed after removal of primary tumors as adjuvant therapy efficiently diminished outgrowth of recurrent primary lesions as well as formation of liver metastases [47].

Overall, this study contributes to the understanding of the influence of the hepatic microenvironment on disseminated PDECs concerning CSC- and EMT-characteristics. Physiological conditions given by an HSC-enriched liver milieu efficiently regulate stemness and differentiation of PDECs. Together with former studies [33], these findings underscore the importance of the hepatic micromilieu in the outgrowth of liver metastases in PDAC.

MATERIALS AND METHODS

Ethics statement

All animal studies were executed in compliance with European guidelines for care and use of laboratory animals and approved by local authorities (V242-77780/2015 (123-10/11)).

Cell lines and cell culture

All cell lines have recently been authenticated by using STR-analysis.

As a model for premalignant pancreatic ductal epithelial cells, the immortalized human PDEC line H6c7-kras containing a G12V-mutation in the *kras* gene (kindly provided by Prof. M.S. Tsao, Ontario Cancer Institute, Toronto, Canada) was used and grown in H6c7-medium (50% keratinocyte-serum free medium (Gibco Life Technologies, Darmstadt, Germany) and 50% Roswell Park Memorial Institute (RPMI) 1640 (Biochrom, Berlin, Germany) supplemented with 5% fetal calf serum (FCS), 0.5% L-Glutamine (both Biochrom), 50 μ g/ml bovine pituitary extract, 5 ng/ml epidermal growth factor (both Gibco Life Technologies), and 0.5 μ g/ml puromycin (Invitrogen, Darmstadt, Germany)). As a model for malignant and poorly differentiated PDECs, the human Panc1 cell line (purchased from ATCC) and as a model for moderately differentiated PDAC cells, the Panc89 cell line (kindly provided by Prof. T. Okabe, University of Tokyo, Japan) were used. The two PDAC cell lines were cultured in Panc-medium (RPMI 1640 supplemented with 10% FCS, 1% L-Glutamine and 1% sodium pyruvate (Biochrom)).

Murine hepatic stellate cells (M1-4 HSC, in the following termed HSC) were used to model a physiological liver microenvironment. Hepatic myofibroblasts (M-HT, in the following termed HMF) were generated from HSC by TGF- β 1-exposition and were used to mimic an inflamed liver environment [28, 33]. Cells were grown in Mi-Medium (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4.5 g/l D-Glucose and 3.7 g/l NaHCO₃ (Biochrom), 10% FCS, 1% sodium pyruvate and 1% L-Glutamine (Biochrom)). Detailed culture conditions have been described previously [33].

Primary murine hepatocytes were isolated as described [48] and grown in hepatocyte-medium (Williams' Medium E (Biochrom) supplemented with 10% SeraPlus (PAN Biotech, Aidenbach, Germany), 1% L-Glutamine, 1% Penicillin/Streptomycin, 2 ng/ μ l Insulin-transferrin-sodium selenite supplement 100 $\times$ (Sigma-Aldrich, St. Louis, USA), 10 μ g/ml Gentamicin (Gibco Life Technologies), 100 nM dexamethasone (Sigma-Aldrich) on a collagen layer (Roche Diagnostics, Mannheim, Germany). Hepatocyte vitality varied between 77% and 98% when seeded for coculture experiments. Confirmatory experiments were performed with human hepatocytes (BioreclamationIVT (BIOIVT), West Sussex, UK; M00995-P LOT: MSW, IAN and AFJ), human HSC and human HMF. Human HSC were purchased from provitro AG (Berlin, Germany). Cells were taken into culture at passage 1 and cultured in SteCM Stellate Cell Medium (provitro AG). To generate a stable and reproducible phenotype of quiescent/weakly activated HSC or HMF human HSC were weekly treated with either 5 μ M all-trans-Retinoic acid (Sigma Aldrich) to generate

HSC or 1 ng/ml TGF- β 1 (BioLegend, Koblenz, Germany) to generate HMF. After a time period of minimum 2 weeks, cells were set in coculture with either H6c7-k_{ras}, Panc1 cells or Panc89 cells under the same conditions as with MI1-4HSC or M-HT. Phenotypes of human HSC and HMF have been previously described [33].

Coculture of PDECs and hepatic stromal cells

For indirect coculture experiments, all cells had been seeded and kept in their respective medium overnight, before cocultures were started by inserting ThinCert™ cell culture inserts (pore size 0.4 μ m, Greiner Bio-One, Frickenhausen, Germany) containing PDECs into corresponding wells containing different hepatic stromal cells. Coculture was conducted for 6 days in a mixture of 50% Panc- and 50% Mi-medium. Monoculture of PDECs was performed under comparable conditions. Three hours after seeding 5×10^4 murine or human hepatocytes on a collagen layer in a 6-well plate, cells were washed twice and medium was exchanged to SeraPlus-free hepatocyte-medium. Alternatively, 4.75×10^4 murine or human hepatocytes were mixed with either 0.25×10^4 murine or human HSC (H+5HSC) or HMF (H+5HMF) and were cultured in 50% SeraPlus-free hepatocyte-medium and 50% Mi-medium. One $\times 10^4$ PDECs were seeded in cell culture inserts in their respective medium.

Colony formation assay (CFA)

CFAs were conducted with mono- and cocultured PDECs and clones derived from single cell expansion. Briefly, 400 single cells were seeded in duplicates into a 6-well culture plate in their respective medium. After cultivation for eight to eleven days, colonies were fixed with 4.5% paraformaldehyde for 10 min, stained with 0.1% crystal violet (Merck Millipore, Darmstadt, Germany) for 1 h, washed in water and air-dried. Only colonies containing more than 50 cells were counted and their morphology regarding holo-, mero- and paraclones was assessed [49, 50]. While holoclones consisted of tightly and homogeneously clustered cells with a regular borderline, paraclones displayed dispersed and larger cells with an irregular borderline [50]. Meroclones exhibited an intermediate colony morphology [50].

Single cell cloning and clone expansion

PDECs were harvested with trypsin, either directly from a culture flask or after 6 day-coculture with hepatocytes and 5% HSC (H+5HSC), to generate a cell suspension with 100 cells in 20 ml of the respective cell medium. 200 μ l of this cell suspension were filled into a flat-bottom 96-well plate (Sarstedt, Nuembrecht, Germany) to seed one single cell per well. Three hours after seeding, first detection was performed by scanning the plate in the brightfield channel of a NyONE cell

imager (SynGene GmbH, Elmshorn, Germany). Only wells containing exactly one cell were considered for further analysis and experiments. After 12 days of regular observation, colony types were characterized and monitored until cell confluence. Only clones forming the same morphology of colonies in CFA were considered for further expansion, *in vitro* and *in vivo* experiments. As cell clones were only used for a maximum of 12 passages, cell clones were also expanded from monoculture conditions showing no differences to clones isolated from H+5HSC-enriched coculture (data not shown).

Neutralization of TNF- α by etanercept

For determining the effects of TNF- α on EMT and stemness, 2.5×10^5 (for RNA isolation) or 400 (for CFA) Panc1 holoclone cells were seeded in Panc-medium in 6-well plates and allowed to attach overnight. Ten μ g/ml Etanercept (Pfizer Deutschland, Berlin, Germany) were applied for 72 h before total RNA was retrieved. Control cells were cultured under comparable conditions without the addition of Etanercept. For CFAs, colonies were grown for 11 days with a second addition of 10 μ g/ml Etanercept on day 7.

RNA isolation and RT-qPCR

Total RNA was isolated using the total RNA kit peqGOLD (PeqLab, Erlangen, Germany) and subjected to reverse transcription according to manufacturer's instructions (Fermentas via Thermo Fisher Scientific, Darmstadt, Germany). qPCR analysis was performed as duplicate analysis on a LightCycler 480 (Roche) including melting curve analysis as quality control. Primers (Eurofins, Ebersberg Germany; RealTime Primers via Biomol, Hamburg, Germany), primer sequences and annealing temperatures are listed in Table 1.

Western blot

Preparation of whole cell lysates and western blot analysis were performed as previously described [51]. The following primary antibodies were used at 1:1000 dilution: anti-E-cadherin (32A8; Cell Signaling, Frankfurt, Germany), anti-HSP 90 α / β (F-8; Santa Cruz, Heidelberg, Germany), anti-L1CAM (9.3; kindly provided by Prof. G. Moldenhauer, German Cancer Research Center, Heidelberg, Germany), anti-Zeb1 (Novus Biologicals, Wiesbaden-Nordenstadt, Germany). Anti-Vimentin (V9; Santa Cruz) was applied 1:200 and anti-phospho-p65 (12H11; Merck Millipore) 1:500. HSP90 was used as loading control. Secondary antibodies anti-mouse IgG (HRP-linked) and anti-rabbit IgG (HRP-linked) (both Cell Signaling) were used at 1:2000 dilution. Primary antibodies were incubated at 4° C overnight, while secondary antibody incubation was performed for 1 h at room temperature (RT). Blots were incubated in Clarity Western ECL Substrate (Bio-Rad Laboratories, München,

Table 1: Primers, their sequences and annealing temperatures used for RT-qPCR

Primer		Primer sequence (5'-3')	Annealing temperature (° C)	Manufacturer
E-cadherin	forward	TGCTCTTGCTGTTTCTTCGG	55	RealTimePrimers
	reverse	TGCCCCATTCGTTCAAGTAG		
GAPDH	forward	TCCATGACAACCTTTGGTATCGTGG	58	Eurofins
	reverse	GACGCCTGCTTCACCACCTTCT		
L1CAM	forward	GAACTGGATGTGGTGGAGAG	58	RealTimePrimers
	reverse	GAGGGTGGTAGAGGTCTGGT		
Nanog	forward	ACCTACCTACCCCAGCCTTT	58	RealTimePrimers
	reverse	CATGCAGGACTGCAGAGATT		
Nestin	forward	GAAACAGCCATAGAGGGCAAA	58	Eurofins
	reverse	TGGTTTTCCAGAGTCTTCAGTGA		
TNF-α	forward	TCCTTCAGACACCCTCAACC	58	Eurofins
	reverse	AGGCCCCAGTTTGAATTCTT		
Vimentin	forward	TCCAAGTTTGCTGACCTCTC	58	RealTimePrimers
	reverse	TCAACGGCAAAGTTCTCTTC		
Zeb1	forward	TCCATGCTTAAGAGCGCTAGCT	61	Eurofins
	reverse	ACCGTAGTTGAGTAGGTGTATGCCA		

Germany) and visualization of proteins was performed by using the Fusion SL detection system (Vilber Lourmat, Eberhardzell, Germany).

Immunofluorescence staining of cells grown on cover slips

Cells grown confluent on cover slips were fixed in 4.5% paraformaldehyde for 10 min and permeabilized with methanol for 10 min at -20°C . Blocking was performed using 4% Bovine Serum Albumin (BSA, Serva Electrophoresis, Heidelberg, Germany) in PBS supplemented with 0.3% Triton X-100 (Sigma-Aldrich) for 1 h at RT, before primary antibody incubation was performed at 4°C in a humidified chamber overnight. Antibodies were diluted in 1% BSA/PBS/0.3% Triton X-100 as follows: anti-Nanog (D73G4; Cell Signaling) 1:100, anti-Nestin (10C2; Thermo Fisher Scientific) 1:200 and anti-E-cadherin (32A8; Cell Signaling) 1:50. After washing, incubation with fluorochrome-labeled secondary antibodies (AlexaFluor 488 anti-mouse; AlexaFluor 546 anti-rabbit (both Life Technologies, Carlsbad, USA), diluted 1:500 in 1% BSA/PBS/0.3% Triton) and 2 $\mu\text{g}/\text{ml}$ Hoechst 33258 (Sigma-Aldrich) for nuclear staining was performed for 1 hour at RT before samples were mounted in FluorSave Reagent (Merck Millipore) mounting medium. Control staining was performed in parallel with isotype control antibodies (Isotype control mouse IgG1

(MAB002; R&D Systems, Minneapolis, USA); Isotype control rabbit IgG (SP137; abcam, UK)) and showed no or only weak staining.

Tumorigenicity assay *in vivo*

All animal studies were executed in compliance with European guidelines for care and use of laboratory animals and approved by local authorities (V242-77780/2015 (123-10/11)).

One $\times 10^4$ Panc1 holoclone or paraclone cells, derived from single cell expansion after HSC-enriched coculture and diluted in 75 μl PBS, were intrasplenically inoculated in 8-week old, female SCID-beige mice (each group $n = 10$) (Charles River, Sulzfeld, Germany). Progression of tumor formation was monitored regularly by palpation and abdominal ultrasound examination using Vevo 770 (FUJIFILM VisualSonics Inc., Toronto, Canada). After 145 days, mice were sacrificed and pancreata, livers and lungs were fixed in 4.5% PBS-buffered formalin and embedded in paraffin before sectioning (2–3 μm) and immunohistological examination.

Immunofluorescence and immunohistochemical stainings of paraffin-embedded tissue sections

Formalin-fixed, paraffin embedded tissue sections were deparaffinized and rehydrated by xylene and

decreasing alcohol concentrations. For immunofluorescence stainings, antigen retrieval was performed with citrate buffer pH 6.0 at sub-boiling temperature for 20 min before blocking, antibody incubation and mounting were performed as described above. Additionally, Sudan Black B treatment (0.1% in 70% Ethanol, Sigma-Aldrich) was performed for 20 min at RT preceding secondary antibody incubation. For immunohistochemical stainings, primary antibody anti-human Cytokeratin (Immunotec, Prague, Czech Republic) was applied 1:100. Anti-human/murine E-cadherin antibody was purchased from Dako-Agilent (Santa Clara, USA) and diluted 1:200 in Dako Antibody Diluent M3612. In preparation for antigen retrieval of immunohistochemical stainings, sections were heated overnight in Dako Antigen Retrieval Solution S 1699 in an 85° C water bath. Antigen retrieval for N-cadherin immunohistochemistry was achieved by treating the sections with Fast Enzyme (Zytomed, Berlin, Germany) for 5 min at room temperature. The anti-human N-cadherin antibody 18203 (abcam, Cambridge, UK) was used in a 1:50 dilution. Antigen retrieval for integrin alpha V was achieved by boiling the sections for 10 min in Dako Antigen Retrieval Solution S 1699 and treating them with an Integrin alpha V antibody (abcam # ab150361). For Vimentin immunohistochemistry, antigen retrieval was achieved by treating the sections for 20 min in a Dako S2367 pH9 antigen retrieval solution. The anti-Vimentin antibody (Dako M7020) was diluted 1:232 in Dako antibody diluent. CD44 was detected after antigen retrieval at 80 C for 10 min in a citrate buffer pH6 and application of the anti-CD44 antibody (BD Pharmingen 550392, Heidelberg, Germany) at a dilution of 1:100 in Dako antibody diluent. The antigen binding sites were detected by using ABC streptavidin complexes with alkaline phosphatase as the marker enzyme (Vectorlabs, Burlingame, USA).

Statistical analysis

Statistical analysis was performed using SigmaPlot 12.5 (Systat, Erkrath, Germany). When comparing two groups with parametric datasets, paired *t*-tests were executed. For multiple groups with parametric data sets, repeated measures analysis of variance (one-way RM ANOVA) test was performed, while non-parametric data were analyzed with the Kruskal-Wallis one-way ANOVA on ranks test. Statistical significances were detected by Tukey test or Student-Newman-Keuls method with *p*-values beneath 0.05 regarded as statistically significant and marked by asterisks (*).

Abbreviations

CFA: Colony formation assay; CSC: Cancer stem cell; EMT: Epithelial-Mesenchymal-Transition; H:

Hepatocyte; HMF: M-HT; HMFs: Hepatic myofibroblasts; HSC: M1-4 HSC; HSCs: Hepatic stellate cells; MET: Mesenchymal-Epithelial-Transition; PDAC: Pancreatic ductal adenocarcinoma; PDECs: Pancreatic ductal epithelial cells

Author contributions

H.K. performed the majority of experiments, L.L., F.V., S.R., L.-M.P. and L.M. contributed to coculture experiments and stem cell analysis. C.H., J.-H.E. and J.-P.G. performed animal experiments, O.W. and S.T. performed the imaging in animal experiments. U.S. performed immunohistochemical stainings. W.M. isolated, characterized and provided M1-4 HSC and M-HT cells. J.G.H. isolated, characterized and provided hepatocytes. S.Se. conceived the study and provided resources. H.K. and S.Se. wrote the manuscript. All authors read and approved the final version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Review

The Heterogeneity of the Tumor Microenvironment as Essential Determinant of Development, Progression and Therapy Response of Pancreatic Cancer

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Simple Summary: Pancreatic ductal adenocarcinoma (PDAC) is still one of the deadliest cancers in western countries. It is commonly diagnosed at advanced stages and most anti-cancer therapies have failed to substantially improve prognosis of PDAC patients. PDAC is characterized by a profound inflammatory tumor microenvironment (TME) comprising various non-neoplastic cells e.g., myofibroblasts, macrophages, T cells and endothelial cells which can exhibit different functional phenotypes. Furthermore, the microbiome is altered in the tumor and other body compartments of PDAC patients adding to the great TME heterogeneity and its impact on PDAC development, progression and therapy responses. This review summarizes the recent knowledge on the diverse phenotypes of these different stromal components. A better understanding of tumor cells as well as TME heterogeneity and considering tumor-suppressing and tumor-promoting phenotypes might provide an important step towards a more effective treatment for this highly malignant tumor.

Abstract: Pancreatic ductal adenocarcinoma (PDAC) is commonly diagnosed at advanced stages and most anti-cancer therapies have failed to substantially improve prognosis of PDAC patients. As a result, PDAC is still one of the deadliest tumors. Tumor heterogeneity, manifesting at multiple levels, provides a conclusive explanation for divergent survival times and therapy responses of PDAC patients. Besides tumor cell heterogeneity, PDAC is characterized by a pronounced inflammatory stroma comprising various non-neoplastic cells such as myofibroblasts, endothelial cells and different leukocyte populations which enrich in the tumor microenvironment (TME) during pancreatic tumorigenesis. Thus, the stromal compartment also displays a high temporal and spatial heterogeneity accounting for diverse effects on the development, progression and therapy responses of PDAC. Adding to this heterogeneity and the impact of the TME, the microbiome of PDAC patients is considerably altered. Understanding this multi-level heterogeneity and considering it for the development of novel therapeutic concepts might finally improve the dismal situation of PDAC patients. Here, we outline the current knowledge on PDAC cell heterogeneity focusing on different stromal cell populations and outline their impact on PDAC progression and therapy resistance. Based on this information, we propose some novel concepts for treatment of PDAC patients.

Keywords: PDAC; tumor stroma; immune cells; endothelial cells; CAFs; myofibroblasts; microbiome; macrophages; personalized medicine

1. Clinical Situation and Challenges

Pancreatic ductal adenocarcinoma (PDAC) is the 4th most frequent cause of cancer related deaths in western countries [1]. Since 80% of PDAC are diagnosed at advanced tumor stages, curative treatment options are limited. Accordingly, the overall 5-year survival rate is still less than 10% [1]. Besides late diagnosis, an early metastatic dissemination as well as a profound therapy resistance contribute to the clinical challenges in the treatment of PDAC and thereby to the patient's dismal situation [2,3].

Originating from pancreatic ductal epithelial cells (PDEC) or pancreatic acinar cells, PDAC can develop via different precursor lesions, such as pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN), mucinous cystic neoplasm, or other lesions [4], to an invasive carcinoma with the liver being the main site of metastasis [5,6]. During this multi-step progression various epi-/genetic alterations are acquired e.g., mutations in the oncogene *KRAS* which is one of the earliest genetic alterations and found in 99% of even early low-grade PanIN [7,8]. Besides genetic and epigenetic changes in PDEC and later on PDAC cells, the adjacent microenvironment also undergoes considerable alterations. These involve enrichment of different leukocyte cell populations (e.g., monocytes, lymphocytes), activation and modification of tissue resident cells (e.g., fibroblasts/stellate cells, endothelial cells), remodeling of the extracellular matrix (ECM) and changes of the microbiome [9–11]. Accordingly, composition and proportion of the microenvironment in close vicinity to precursor/PDAC cells are highly variable as they evolve concomitantly with PDAC progression. Furthermore, the TME is mutually dependent on both environmental factors (e.g., aging, exposure to lifestyle factors) and the (epi-)genetic make-up of PDEC and PDAC cells, respectively [12]. This indicates a dynamic co-evolution of neoplastic and non-neoplastic cells during pancreatic tumorigenesis being characterized and driven by a high heterogeneity of both different tumor cell clones and stromal cell populations. PDAC is characterized by a pronounced inflammatory tumor microenvironment (TME), which often accounts for the major tumor mass. Therefore, it is not surprising that therapies targeting genetically related alterations (e.g., altered signaling by mutant *KRAS* or sustained Epidermal Growth Factor Receptor (EGFR)-signaling caused by *EGFR* overexpression) have failed to improve treatment of PDAC patients as the blocked signaling pathways are bypassed by the stroma-mediated signaling which further sustains the cellular processes in the transformed cells [13,14]. Similarly, chemotherapeutic regimens have largely failed to improve the prognosis of PDAC patients because PDAC cells exhibit multiple strategies by which they evade the effect of cytostatic drugs. Importantly, these strategies are promoted by the stromal cells and additionally, the TME itself impairs the drug efficacy of PDAC (e.g., by metabolism of cytostatic drugs) [15–18]. In this context, the microbiome seems to play an important role, too. Several studies have already shown that the gut and tumor microbiome are altered in PDAC patients, promoting tumor progression and reducing treatment responses of PDAC cells, thereby substantially impairing survival of PDAC patients [11,19,20].

Finally, immune checkpoint inhibitors have revolutionized the treatment of various solid tumors even at advanced stages such as non-small cell lung carcinoma but have failed in the treatment of PDAC patients [21–23]. Besides a heterogeneous expression pattern of the immune checkpoint regulator programmed cell death 1 ligand 1 (PD-L1) on PDAC cells [24], another reason might be the high heterogeneity of the stromal compartment not only with respect to PD-L1 expression, but also regarding the cellular composition (inflamed versus non-inflamed tumors) [25,26].

Thus, experimental and clinical data strongly support the view that the failure of current therapeutic strategies is due to an insufficient consideration of the heterogeneity of

PDAC cells but also stromal cells. In order to improve our understanding of this heterogeneity, we herein outline the recent knowledge on TME diversity and its impact on PDAC development, progression and therapy resistance. Being aware of the numerous publications in this field in recent years, we apologize for only citing a selection. Furthermore, considering this knowledge we propose some concepts for novel therapeutic strategies to improve prognosis of PDAC patients.

2. Heterogeneity of the Tumor Cell Compartment in PDAC

Comprehensive (single cell) omics analyses revealed a high diversity of tumors at multiple levels, namely interpatient, intertumor and intratumor heterogeneity altogether providing an explanation for the noticeable differences in therapy responses and survival of cancer patients [27]. In stroma-rich PDAC, those analyses were combined with microdissection allowing a distinct investigation of tumor and stromal cells. In recent years, mainly three studies revealed substantial insights into interpatient heterogeneity. Collisson et al. classified PDAC into “classical”, “quasi-mesenchymal” and “exocrine-like” subtypes. Besides differences in the expression profile, the classical phenotype was an independent prognostic factor for longer overall survival of resected PDAC patients and classical PDAC lines were more resistant towards Gemcitabine treatment than cell lines with a quasi-mesenchymal phenotype [28].

Moffitt et al. identified two tumor cell subtypes (“basal-like” and “classical”, the latter one overlapping with those identified by Collisson et al.) but additionally two stromal subtypes (“normal” and “activated”). Here, patients with a tumor of the “basal-like” subtype or exhibiting an activated stroma had a worse prognosis compared to either corresponding subgroup. However, basal-like tumors seemed to show better responses towards adjuvant therapy. Importantly, the normal stroma signature was associated with high expression of genes typical for pancreatic stellate cells (PSC), while an activated stroma signature was characterized by high expression of genes related to macrophages and myofibroblasts, both being abundant in the PDAC stroma [29].

Finally, Bailey et al. identified four subtypes, namely “squamous”, “pancreatic progenitor”, “immunogenic” and “aberrantly differentiated endocrine exocrine (ADEX)”. These subtypes clearly differed with respect to genetic and epigenetic alterations as well as transcription profiles. Additionally, the “immunogenic” subtype was characterized by an enrichment of different immune cell subsets and enhanced immune evasion pathways e.g., mediated by the immune checkpoint regulators Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4) and Programmed cell death protein 1 (PD-1) [30].

Extending these findings, Birnbaum et al. compared the tumor subtypes identified by Collisson, Moffitt and Bailey and confirmed the independent prognostic value of the classification from the latter two studies [31]. Additionally, Rashid et al. provided evidence that a tumor classification into two subtypes (basal-like and classical) according to Moffitt et al. exhibits the highest robustness and clinical relevance by demonstrating that basal-like tumors showed a strong resistance to FOLFIRINOX compared to classical tumors [32]. Although all studies revealed important insights into interpatient tumor heterogeneity, prospective trials are still needed to validate the clinical utility of this tumoral subtyping in particular with respect to optimized treatment.

With respect to intratumor heterogeneity, it is assumed that phenotypic and genotypic diversity of tumor cell clones within (primary and secondary) tumors originate from clonal expansions and differentiation hierarchies according to the current model of cancer stem cells (CSC) [33,34]. Thus, intratumor heterogeneity can occur and manifest on multiple levels based on the expansion of tumor cell clones with different genetic (driver as well as passenger mutations) and epigenetic alterations (hypermethylated DNA regions, histone modifications) as well as divergent differentiation stages (CSC versus non-CSC stage). These alterations in turn essentially contribute to different metabolic requirements (dependence on glucose, lactate or glutamine) as well as phenotypic diversity within the tumor mass resulting in e.g., proliferating versus resting, sessile versus motile/invasive

or therapy responsive or resistant tumor cell clones [35,36]. Important to note, expansion and evolution of certain tumor cell clones are highly dependent on the adjacent stroma which co-evolves with the different tumor cell clones during pancreatic tumorigenesis, applying to the primary as well as to the secondary context [37,38]. Although it can be speculated whether the metastatic tumor stroma induces the above-mentioned epigenetic, metabolic and thereby phenotypic alterations or selects for those clones that have acquired these alterations before leaving the primary tumor, a fundamental role of the stromal compartment in this heterogeneity determining process is meanwhile indisputable.

Thus, it is necessary to unravel all levels of tumor diversity and in particular stromal cell heterogeneity (Figure 1) to gain a complete picture of PDAC complexity which has to be considered for optimized treatment strategies.

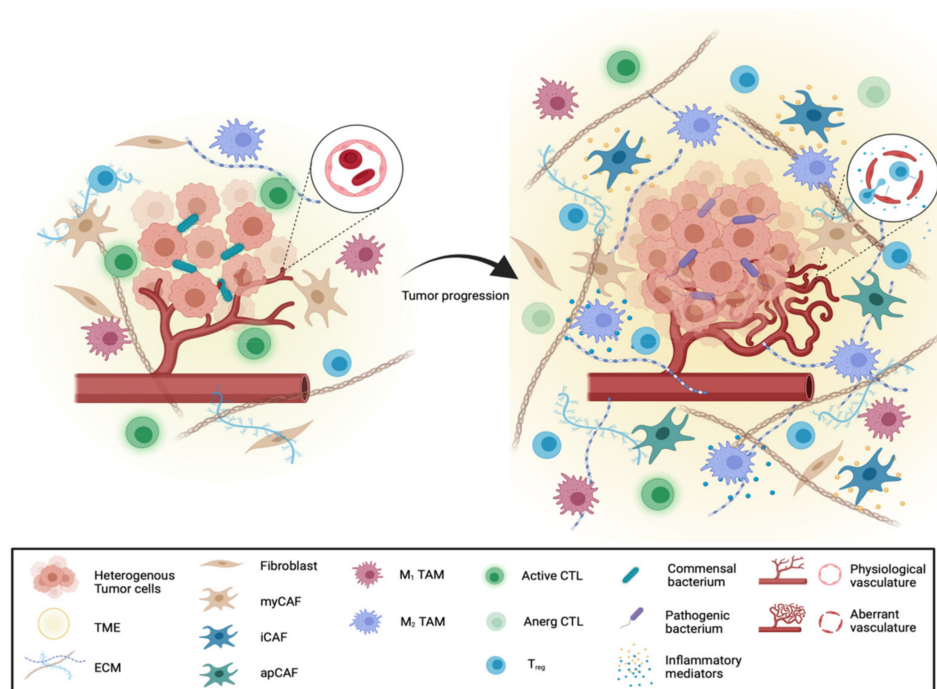


Figure 1. Pancreatic cancer is characterized by a high tumor and stroma heterogeneity. During pancreatic tumorigenesis, tumor cells acquire multiple alterations resulting in a heterogeneous pool of tumor cell clones. Along with the evolution of these divergent tumor cell clones, the tumor microenvironment (TME) also undergoes fundamental alterations involving, (i) activation of fibroblasts and transdifferentiation into carcinoma associated fibroblasts (CAFs) with different phenotypes (myCAF, iCAF, apCAF), (ii) alteration of the extent and composition of the extracellular matrix (ECM), (iii) enrichment of immunosuppressive and tumor-promoting immune cells e.g., tumor-associated macrophages with different phenotypes (M1 TAM versus M2 TAM), anergy cytotoxic T cells (CTL) and regulatory T cells (Treg), (iv) enrichment of pathological bacteria and extrusion of commensal bacteria, (v) elevation of inflammatory mediators and (vi) formation of new and modified blood vessels. Overall, this TME heterogeneity is an essential driver of PDAC development and progression. Figure created with [BioRender.com](https://www.biorender.com).

3. Heterogeneity of the Stromal Compartment in PDAC

3.1. Heterogeneity of Carcinoma Associated Fibroblasts

The most abundant inflammatory stroma cells in PDAC are myofibroblasts, also termed carcinoma associated fibroblasts (CAF). CAF are abundant in primary PDAC as well as in metastases e.g., in the liver. During cancer development, several cell types can transdifferentiate into CAF. Besides PSC, adipocytes, monocytes, bone marrow-derived mesenchymal stem cells and endothelial cells (EC) can give rise to CAF in the pancreas [39–43]. In the liver, the main

source of CAF is hepatic stellate cells (HSC), with CAF also originating from hepatic sinusoidal endothelial cells or portal mesenchymal cells [44–50].

Under physiological conditions, most pancreatic fibroblasts are a heterogeneous group of desmin+ PSC which produce and maintain physiological levels of ECM proteins such as collagen IV, laminin, fibronectin and glycosaminoglycans. Thereby, these cells essentially contribute to the physiological pancreas homeostasis [51–56]. PSC are located at the base of the acini and contain high amounts of vitamin A vesicles [51]. Upon activation PSC lose these lipid vesicles and transdifferentiate into myofibroblasts, thereby increasing their proliferative and migratory potential, suppressing pro-apoptotic gene expression and secreting cell cycle promoting proteins [57–59]. Furthermore, myofibroblasts produce elevated amounts of ECM proteins at an altered composition, resulting in fibrosis. This fibrotic ECM is composed of less elastin and more fibronectin as well as crosslinked collagen type I and III [60]. Baron et al. distinguished two groups of pancreatic myofibroblasts: The first phenotype is characterized by production of ECM, and the second phenotype, called immune-activated PSC, exhibits and releases high expression levels of immunomodulating cytokines, interleukins and chemokines (e.g., chemokine (C-X-C motif) ligand 3 (CXCL3), Interleukin-33 (IL-33), IL-2) [61]. Exposure to stressful conditions including exogenous toxins, surgical intervention, inflammatory injury or mediators released by immune cells [56,62,63] promotes proliferation and activation of PSC into myofibroblasts leading to increased alpha-smooth muscle actin (α -SMA) expression. Stimuli leading to the transdifferentiation and activation of myofibroblasts/CAF are Transforming Growth Factor-beta (TGF- β), Platelet-derived Growth Factor (PDGF) and IL-6 [64–67]. Meanwhile, it is well known that CAF can exhibit different phenotypes and different CAF populations have been discovered in recent years. In the pancreas, major subgroups are myofibroblastic CAF (myCAF), inflammatory CAF (iCAF) and major histocompatibility complex II (MHCII) antigen presenting CAF (apCAF) [68]. MyCAF, which are tumor-adjacent and express high levels of α -SMA, are activated in a TGF- β dependent manner [69,70]. In contrast, iCAF are localized distant from the tumor, express low α -SMA levels and exhibit rather tumor-promoting and immunosuppressive properties by secreting inflammatory cytokines, like IL-6, CXCL12 and Granulocyte-Colony stimulating Factor (G-CSF). In contrast to myCAF, iCAF differentiation is stimulated by cancer-secreted IL-1 via the Januskinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway and antagonized by TGF- β . In vitro, PSC can differentiate into either myCAF or iCAF and even after this differentiation, iCAF can transdifferentiate into tumor-suppressive myCAF [68,69]. These findings strongly support the existence of different (functional) CAF phenotypes which seem to be interchangeable and highly context dependent. In both murine and human tissues, Hutton et al. recently identified two further functionally distinct pancreatic fibroblast populations by cluster of differentiation 105 (CD105) expression. While CD105+ fibroblasts did hardly impact tumor growth in vivo, CD105- fibroblasts promoted anti-tumor immunity and suppressed tumor growth. Characteristics of the established myCAF and iCAF populations were found in either lineage, whereas all apCAF were CD105-. In contrast to myCAF and iCAF populations, CD105+ and CD105- pancreatic fibroblasts seemed not to be interconvertible [71].

As outlined above, CAF are the major source of ECM in the TME. Cross-linked and oriented collagens cause a stiffened matrix, which stimulates accelerated cell cycle progression in adjacent tumor cells and thus fuels tumor growth [72]. Besides, the dense stroma acts as a physical barrier for drugs and immune cells [69]. Neumann et al. showed that the response to chemotherapeutic treatment (e.g., Gemcitabine, Nab-paclitaxel) is reduced when tumor cells are directly cocultured with human CAF compared to monocultured tumor cells [73]. Furthermore, T cells are unable to infiltrate into the dense matrix, which might be one reason for the limited clinical success of immunotherapy in PDAC patients [74]. By compressing blood and lymphatic vessels, perfusion is impaired and the interstitial fluid pressure increased, which induces hypoxia mediated progression and promotes cancer invasiveness, respectively [75–78]. Hypoxia in turn selects the most malignant

cancer cells and through hypoxia-inducible factor 1 (HIF-1) suppresses tumor suppressor genes while promoting the metastatic cascade [79–82]. By promoting M2-like polarization in macrophages and increasing PD-L1 expression in cancer cells, tumor-associated macrophages (TAM) and dendritic cells (DC), HIF-1 supports immunosuppression in the TME [83–87].

Besides the ECM-mediated effects, CAF exert several direct effects on the tumor. Thus, CAF promote an immunosuppressive TME by selecting tumor-promoting immune cells and inhibiting tumor-suppressive ones. Hence, in a KPC mouse model, increased PD-L1 and PD-L2 expression was demonstrated on CAF compared to fibroblasts [88]. Multiple murine and human studies showed their capability of excluding cytotoxic T cells (CTL) from tumor islands and inhibiting T cell activity in multifaceted ways [88–91]. In a mouse model, Feig et al. found CAF-derived CXCL12 to coat PDAC cells and protect them from CD8⁺ T cell invasion [90]. Lakins et al. showed murine CAF to cross-present tumor-derived antigens on MHC I and upon contact with CTL, induce cell death via PD-1/PD-L2 and Fas/FasL interaction [91]. While preventing CTL functions, CAF recruit immunosuppressive immune cells, such as myeloid derived suppressor cells (MDSCs) and monocytes, and promote monocyte differentiation into M2-like macrophages via Macrophage-Colony Stimulating Factor (M-CSF) [92–96]. MDSC-promoting factors secreted by CAF include IL-6, Vascular Endothelial Growth Factor (VEGF) and M-CSF [97]. In PDAC, iCAF have been identified to be the major source of these immunomodulating ligands, which might explain their above-mentioned localization at the edge of the tumor [24,70,98–100]. Another CAF subpopulation found in murine and human PDAC and breast cancer are apCAF [101], which are characterized by expression of MHC II molecules and their capability to present antigens to CD4⁺ T cells. However, apCAF lack costimulatory molecules required to promote T cell proliferation. Thus, MHC II molecules on apCAF were hypothesized to act as decoy receptors and exert immunosuppression via induction of anergy in T cells or transdifferentiation into regulatory T cells (Tregs) [100]. Furthermore, CAF can enhance cell growth [102], induce Epithelial-Mesenchymal-Transition (EMT) thereby facilitating PDAC cell dissemination and metastasis and promote an apoptosis and drug resistant phenotype in PDAC as well as precursor cells in a paracrine manner [17,18,103,104].

The mechanisms of myofibroblasts/CAF generation and their functional impact on tumorigenesis apply also to HSC and the derived hepatic myofibroblasts (HMF) [105–107]. HSC make up 5% of all liver cells and share many properties with PSC [108,109]. In the physiological liver, HSC perform a variety of tasks to maintain organ homeostasis by paracrine secretion of hepatocyte mitogens such as Hepatocyte Growth Factor (HGF) and Epidermal Growth Factor (EGF), and they store 50–80% of all retinoids in the body [110–115]. In contrast in the inflamed liver, HMF essentially impact immune responses inducing infiltration of neutrophils and lymphocytes or by acting as professional antigen presenting cells (APC), while also inhibiting T cells via the expression of PD-L1 [116–120]. In a KPC mouse model, Costa-Silva et al. [105] demonstrated that PDAC-derived circulating exosomes are taken up by Kupffer cells inducing TGF- β expression in these liver resident macrophages. TGF- β in turn activates HSC to HMF leading to an increased production of fibronectin and collagen I and III. This pre-metastatic niche was shown to be crucial for the onset of PDAC metastases [105,121–124]. Recently, Bhattacharjee et al. also identified different CAF populations in murine and human liver tissues based on subpopulations previously characterized in pancreatic and breast cancer. Thus, most liver CAF exhibit a myCAF-like expression profile with high collagen and α -SMA levels, while a smaller subpopulation expresses less α -SMA, but more growth factors and cytokines, and is therefore defined as iCAF [125]. Besides the role in immunomodulation and ECM remodeling, HSC and HMF differentially impact cell growth of PDAC cells, thereby controlling outgrowth of liver metastases. Accordingly, Lenk et al. showed that both murine and human HSC inhibit PDEC and promote a dormant phenotype in an IL-8 dependent manner, while HMF promote reawakening of the dormant stage and enhance proliferation in a VEGF dependent fashion [106].

3.2. Approaches for Targeted Therapy of Carcinoma Associated Fibroblasts

In light of the broad impact of CAF on PDAC progression, immune modulation and drug response, targeting CAF, CAF-derived factors or factors leading to their generation have emerged as attractive targets for PDAC therapy (Table 1).

TGF- β , produced by CAF and being their main activator, enhances tumor progression and dampens the immune and therapy responses [126,127]. Thus, it represents a promising target to interfere with CAF function [128]. However, an early phase clinical trial testing the therapeutic efficacy of pharmacological TGF-blockade with the small molecule inhibitor Galunisertib in combination with chemotherapy for the treatment of unresectable PDAC did not reveal convincing results [129]. Additionally, data from a phase Ib clinical trial investigating Galunisertib in combination with the anti-PD-L1 antibody Durvalumab did not yield clinical improvements for patients with metastatic PDAC [130]. Of note, Özdemir et al. showed in a PDAC mouse model that complete depletion of α -SMA+ myofibroblasts led to enhanced tumor growth, decreased immune cell infiltration and increased number of Treg in the TME. These findings indicate that complete depletion of CAF is not a reasonable treatment strategy, as tumor-restraining CAF populations are eliminated, too. However, concomitant depletion of CAF and blocking of CTLA-4 decreased tumor growth and prolonged survival of PDAC bearing mice [131].

Furthermore, it has been shown that tumor derived ligands of the Sonic Hedgehog (Shh) signaling system leads to activation of stellate cells into CAF [132]. Based on this finding, Shh inhibitors were tested in preclinical and clinical trials to improve PDAC treatment. However, depletion or pharmacological inhibition of Shh reduced stroma formation but accelerated tumor growth of tumor bearing KPC mice [133]. In line with this finding, combined treatment of metastatic PDAC patients with Gemcitabine and Shh inhibitors, targeting the signal transducing component Smoothened (SMO), showed no superior effect compared to Gemcitabine monotherapy [134]. Another clinical trial investigated the efficacy of the SMO inhibitor Saridegib (IPI-926) in combination with FOLFIRINOX for treatment of patients with advanced PDAC demonstrating an antitumor activity along with acceptable safety [135]. However, since a study demonstrated that patients being treated with IPI-926 in combination with Gemcitabine showed even shorter survival times than patients treated with Gemcitabine alone, this study was terminated prematurely (see Infinity Pharmaceuticals company website) and other trials investigating the therapeutic efficacy of Hh inhibitors were withdrawn or stopped.

Due to the tumor boosting effect, achieved by depleting the entire CAF population, recent research has rather focused on stromal reprogramming than on stroma depletion by e.g., reversal of CAF activation or shifting the CAF populations. Since activated myofibroblasts can be reversed into a quiescent phenotype by retinoic acid [136], all-trans retinoic acid (ATRA) has been used for myofibroblast reversal. Thus, ATRA treatment of tumor bearing KPC mice resulted in an enhanced CTL infiltration in the tumor tissue, which was explained by reduced secretion of the chemokine CXCL12 by reverted stellate cells [88]. Another strategy aiming at a stromal (CAF) reprogramming is the treatment with Calcipotriol, which is a derivative of vitamin D. The vitamin D receptor itself is a transcriptional factor suppressing the activation of PSC. Treatment with Calcipotriol led to stroma remodeling and combined treatment with Gemcitabine reduced tumor growth and increased overall survival of PDAC bearing mice [137]. Recently, Biffi et al. demonstrated a phenotypic shift from tumor-promoting iCAF to tumor suppressive myCAF by JAK inhibition, which was associated with reduced tumor growth and increased collagen deposition in tumor bearing KPC mice, further impairing drug penetration into the tumor stroma [70]. This was also seen in clinical trials where the combination of a JAK inhibitor and chemotherapy did not prolong the overall survival [138].

Taken together, CAF represent a heterogeneous cell population in the TME of PDAC at the primary site as well as in metastases exerting diverse functions on other stromal cells (immune cells, EC) and PDAC cells. Importantly, CAF and their quiescent/non-activated counterpart, the stellate cells may exert tumor-restraining functions, which have been

taken into consideration for the development of stroma-targeted therapeutic strategies to improve PDAC treatment.

Table 1. Overview of the cited therapeutic approaches targeting carcinoma associated fibroblasts (CAF).

Study System	Targeting Strategy	Reference
Preclinical studies		
PKT mouse model	Myofibroblast depletion by Ganciclovir	[131]
PKT mouse model	Myofibroblast depletion by Ganciclovir + anti-CTLA-4 antibody	[131]
KPC mouse model	Depletion or pharmacological inhibition of Shh	[133]
KPC mouse model	Myofibroblast reversal by ATRA	[88]
KPC mouse model	Vitamin D derivative Calcipotriol + Gemcitabine	[137]
KPC mouse model	Phenotype shift by JAK inhibitor (AZD1480)	[70]
Clinical trials		
Phase 1b/2 clinical trial	TGF- β blockade by Galusertinib + Gemcitabine	[129]
Phase 1b clinical trial	TGF- β blockade by Galusertinib + PD-L1 antibody Durvalumab	[130]
Phase 1b/2 clinical trial	Shh blockade by Vismodegib + Gemcitabine	[134]
Phase 1 clinical trial	SMO inhibition by Saridegib (IPI-926) + FOLFIRINOX	[135]
Phase 3 clinical trial	JAK1/JAK2 inhibition by Ruxolitinib + Capecitabine	[138]

3.3. Heterogeneity of Macrophages

Besides CAF, macrophages are also found in high numbers in many tumor entities representing the most abundant leucocyte population in the TME [139], also applying to PDAC [140]. Other, but less abundant, myeloid cell populations in PDAC are DC and MDSC. Tjomsland et al. showed that increased numbers of DC in the peripheral blood are associated with a better survival in PDAC patients [141]. However, most human PDAC specimens are characterized by low numbers of DC [142] and even if DC are present, they are mostly located at the tumor margins [142], a finding which could be similarly found in a modified KPC mouse model [143]. DC comprise a variety of subtypes including conventional and plasmacytoid DC exhibiting distinct phenotypes [144]. Of note, DC are key APC for the activation of T cells. Accordingly, these cells essentially determine the efficacy of anti-tumor immunosurveillance and immunotherapeutic treatment strategies, such as immune checkpoint inhibition and vaccinations. As described for other stromal cell populations, also MDSC can exhibit different phenotypes. In humans, three major MDSC subsets were identified: polymorphonuclear (PMN)-MDSCs, monocytic (M)-MDSCs and “early-stage MDSCs” (e-MDSC) [145]. Trovato et al. demonstrated that the MDSC frequency in PDAC was significantly correlated with overall survival and metastatic disease in PDAC patients, but only in some patients the immunosuppressive activity of purified MDSC was detectable. However, this finding was ascribed only to the monocytic subset [146]. One reason for the activity and expansion of MDSC in PDAC seems to be CD200, a regulator of myeloid cell activity, which is expressed in the PDAC TME. Choueiry et al. showed that MDSC isolated from human PDAC tissues express elevated levels of the CD200 receptor, by which CD200 can activate MDSC. Additionally, they found in a mouse model that the CD200 blockade impaired tumor progression and enhanced the efficacy of PD-1 immune checkpoint inhibition [147] supporting the immunosuppressive nature of these cells. Besides, it was shown that MDSC decrease T cell proliferation and lead to an enhanced apoptosis of activated T cells [148]. However, the exact mechanisms by which

MDSC exert their immunosuppressive functions are still not fully elucidated and we refer to other excellent reviews on this cell type [149].

Owing to their prominent abundance in PDAC, we will focus in the following text mainly on the recent knowledge on TAM. TAM mainly originate from tissue resident macrophages or from blood derived monocytes which infiltrate into the injured tissue (e.g., due to cell infection or otherwise induced cell damage). Ontogeny studies in a murine PDAC model revealed that monocyte-derived TAM acquire functions such as antigen presentation, whereas embryonically-derived TAM show a more pro-fibrotic transcriptional profile indicating their role in ECM production and relating the origin of TAM to distinct functional phenotypes [150]. Importantly, in dependence on the environmental factors, monocytes/macrophages acquire different phenotypes exerting distinct functional effects. According to a simplified model, macrophages can differentiate into M1- and M2-macrophages. While interferon-gamma (IFN- γ) and bacterial lipopolysaccharide (LPS) promote polarization of pro-inflammatory M1-macrophages, IL-4, IL-13, IL-10 and TGF- β foster an anti-inflammatory M2-phenotype in humans [151–153]. Thus, the composition of the TME (e.g., oxygen level, amount and type of other stromal or tumor cells) impacts phenotype and effector function of macrophages and promotes the switch from one type to another, a process which also occurs under physiological conditions [154]. As outlined above, CAF promote an immunosuppressive environment e.g., via CXCL12 and M-CSF secretion thereby recruiting monocytes and promoting the accumulation of M2-polarized macrophages [90,155,156]. Moreover, a hypoxic TME provokes an M2-polarization, while an oxygen enriched microenvironment drives M1-polarization [83]. In a murine PDAC model, PDAC cell derived small extracellular vesicles (sEV) covered with Ezrin were shown to foster a polarization towards an M2-phenotype and supporting metastasis formation in the liver [157]. Studies in another PDAC mouse model showed that uptake of PDAC-derived exosomes by Kupffer cells contributes to formation of a pre-metastatic niche in the liver involving the recruitment of bone marrow-derived macrophages [105]. Granulin, expressed by circulating monocytes and hepatic metastasis-associated macrophages, leads to activation of HSC into HMF thereby promoting liver metastasis formation [158]. Thus, CAF and TAM maintain a vicious stimulatory cycle that sustains and further fuels itself, contributing to a tumor-promoting TME.

M1-macrophages secrete different pro-inflammatory cytokines such as IL-12, IL-23 and express elevated levels of B7 family members (B7-1, B7-2) and MHC II for appropriate activation of a TH1 response by which these cells foster anti-tumor effects [159]. In contrast, M2-macrophages secrete anti-inflammatory cytokines such as IL-10, TGF- β and others to prevent T cells from effectively exerting their anti-tumor functions [160].

Although the frequency and phenotype of macrophages are subject to high dynamic variation of the pancreatic microenvironment during tumorigenesis [140], TAM in PDAC predominantly exhibit M2-characteristics. These are associated with diverse pro-tumoral effects including promotion of tumorigenesis, immunosuppression, metastasis acceleration and chemotherapeutic resistance [159]. In primary PDAC, a high infiltration of macrophages, particularly CD163+ macrophages, is observed [24,140,161–163], indicating an M2-type which has been associated with a poor prognosis and decreased survival [162–165]. Additionally, the number of TAM is inversely associated with the maturity of the tumor stroma, as an immature (highly cellular and collagen poor) stroma correlates with an increased the number of TAM [163]. As M2-macrophages secrete a plethora of growth factors, cytokines and chemokines (e.g., IL-10, IL-6, Tumor Necrosis Factor-alpha (TNF- α), TGF- β , chemokine (C-C motif) ligand 20 (CCL20)), they are potent inducers of the EMT [161,166]. Furthermore, PDAC tumor growth and metastasis formation are dependent on phosphatidylinositol 3-kinases (PI3K γ) expression in macrophages [167]. Of note, TAM can co-exist as M1- or M2-macrophages or also intermediate forms in the TME which are associated with anti- or pro-tumoral functions [168]. Accordingly, Helm et al. could demonstrate that human PDAC-derived TAM can exhibit both M1- and M2-properties and that also M1-macrophages are able to increase migratory and invasive abilities of PDAC cells [161,168]. In line with these findings, Chen et al. showed that M2-

polarized macrophages cause inflammation via IL-1 β release. This increased IL-1 β release in turn promotes EMT induction and thereby formation of metastases in an orthotopic PDAC mouse model [169].

Macrophages also contribute to apoptosis and therapy resistance of PDAC. Buchholz et al. showed that murine and human macrophages can rapidly metabolize and inactivate the chemotherapeutic drug Gemcitabine resulting in profound therapy resistance [15]. Another study could show that murine bone marrow derived macrophages when exposed to PDAC cells release pyrimidine species, among others deoxycytidine, which inhibits gemcitabine through molecular competition representing another mechanism of macrophage mediated chemoresistance [170]. Liu et al. suggest that macrophages contribute to immunosuppression and failure of immunotherapies by demonstrating an accumulation of MDSC and M2-polarized TAM in tumoral lesions of different PDAC mouse models which is accompanied by a reduction of CTL and T helper cells. Besides, the number of M2-macrophages increased after application of Gemcitabine in this model system, which could be another mechanism for drug resistance [171]. Weizman et al. identified in humans another TAM-mediated mechanism of chemoresistance via upregulation of cytidine deaminase (CDA) which leads to reduction of Gemcitabine-induced apoptosis. CDA is the enzyme which transports chemotherapeutics into cells and is responsible for their metabolism [172]. Using a genetic mouse model, Binenbaum et al. revealed that TAM communicate with cancer cells via so called macrophage-derived exosomes which are internalized by cancer cells leading to reduced Gemcitabine concentrations by upregulation of CDA in the PDAC cells [173]. Moreover, tumor-derived exosomes can be taken up by human and murine macrophages, thereby increasing expression of the immune checkpoint molecule PD-L1 on these cells and promoting macrophage-mediated immune suppression. Uptake of tumor-derived exosomes also alters the cytokine secretion (IL-6, IL-1 β , IL-10 and TNF α) of macrophages thereby facilitating several pro-tumoral functions [174].

3.4. Approaches for Targeted Therapy of Macrophages

As macrophages exert diverse pro-tumoral effects e.g., promoting an immunosuppressive TME and drug resistance, these stromal cells have been qualified as a reasonable target for cancer therapy (Table 2). Accordingly, several preclinical and clinical trials targeting macrophages have been conducted. One approach to simultaneously harness multiple immune cell types including macrophages against cancer cells is the treatment with CD40 agonists [175]. CD40 is a cell surface receptor primarily expressed by B cells, DC and myeloid cells including macrophages. Through interaction with its ligand CD40L (expressed on CD4+ T cells), CD40 is activated. This activation results in various physiological effects including increased expression of MHC II and T cell costimulatory receptors on its target cells [175,176]. Consequently, antigen presentation and CTL activation are promoted. Moreover, in the KPC mouse model, CD40 agonist treatment resulted in macrophage activation with increased expression of matrix metalloproteinases. Via this mechanism, macrophages reduced the pronounced PDAC-associated desmoplasia [177]. Recently, macrophage-mediated stroma depletion and reprogramming towards an M2-phenotype were confirmed for human PDAC by translational data obtained from a clinical trial that assessed the CD40 agonist Selicrelumab [178]. Moreover, in a PDAC mouse model, CD40 agonists synergized with checkpoint inhibitors [179]. Whilst early clinical trials suggested a positive clinical effect of CD40 agonist monotherapy or combination with Nivolumab [177,180], this could not be confirmed in phase II clinical trials [181]. However, multiple clinical trials evaluating CD40 agonists for the treatment of PDAC in combination with chemotherapeutic or targeted agents are ongoing (NCT03214250; NCT04536077; NCT04807972; NCT04888312) and may yield more promising results. Furthermore, the combination of an anti-PD-1 antibody with Gemcitabine showed a beneficial effect in a murine PDAC liver metastasis model, which was explained by an increase of M1-macrophages and a TH1 response [182].

Table 2. Overview of the cited therapeutic approaches targeting macrophages.

Study System	Targeting Strategy	Reference
Preclinical studies		
KPC mice	CD40 agonist treatment by CP-870,893	[177]
KPC mice	CD11b agonist treatment by ADH-503	[183]
C57BL/6 mice including Batf3 KO, CD40 KO, MyD88 KO, STING KO and IFNAR KO	CD40 agonist treatment by FGK45 combined with anti-PD-1 by RMP1-14 and/or anti-CTLA-4 by 9H10	[179]
Clinical studies		
Phase1 clinical trial	CD40 agonist treatment by Selicrelumab	[178]
Phase 1b clinical trial	CD40 agonist treatment by Sotigalimab in monotherapy or in combination with PD-1 blockade by Nivolumab	[180]
Phase 2 clinical trial	CD40 agonist treatment by Sotigalimab in monotherapy or in combination with PD-1 blockade by Nivolumab	[181]
Phase 1b/2 clinical trial	CD40 agonist treatment by Sotigalimab in combination with PD-1 blockade (Nivolumab) + Gemcitabine + Nab-Paclitaxel, or Sotigalimab + Gemcitabine + Nab-Paclitaxel.	NCT03214250
Phase 2 clinical trial	Addition of recombinant fms-like tyrosine kinase 3 (Flt-3) ligand (CDX-301) to the CD40 agonistic antibody (CDX-1140)	NCT04536077
Phase 2 clinical trial	modified FOLFIRINOX (mFFX) combined with ABBV-927 with or without Budigalimab	NCT04807972
Phase 1b/2 clinical trial	CD40 agonist Mitazalimab in combination with modified FOLFIRINOX	NCT04888312

Since specific targeting of macrophages spares other immunosuppressive monocytic cell populations which may impair the therapeutic efficacy, targeting of the integrin CD11b/CD18 which highly expressed on several myeloid cell subsets has been suggested as a promising strategy for PDAC treatment. Using the CD11b small-molecule agonist (ADH-503) in KPC mice, a partial activation of CD11b along with TAM repolarization was observed. In addition, increased numbers of immunosuppressive myeloid cells in tumor tissues were observed as well as enhanced dendritic cell activity. Thus, this strategy targeting multiple immunosuppressive myeloid cells led to an improved antitumor T cell response which might be even more effective in combination with immune checkpoint inhibitors [183].

However, to date no convincing strategies targeting macrophages have been elaborated and implemented in treatment of PDAC patients. One explanation might be that as demonstrated for CAF, macrophages represent a heterogeneous cell population exerting highly context-dependent pro- and anti-tumoral effects. Thus, this dynamic phenotype

switching, which also seemed to be influenced by the different therapeutic strategies, have to be considered in an effective and sustainable anti-tumor therapy of PDAC patients.

3.5. Heterogeneity of T Cells

Although we focus on T lymphocytes in this article, we want to briefly mention the other group of lymphocytes, namely the B cells. The role of B cells in PDAC is still controversially discussed but in general, B cells are described to be rather tumor promoting. Thus, Pylayeva-Gupta et al. showed that B cells secrete IL-35, which promotes the proliferation of tumor cells [184]. Furthermore, coculture of B cells isolated from PDAC patients enhanced the production of collagen by fibroblasts, resulting in ECM remodeling [185]. In a mouse model, it was shown that B cells are recruited by the B cell chemoattractant CXCL13 already during PanIN formation. Similarly, accumulation of B cells was also observed in PanIN lesions in PDAC patients [184,185]. Overall, there are not a lot of studies focusing on B cells in PDAC but it might be useful to investigate further on B cells in order to understand the complex microenvironment better.

Besides diverse CAF and macrophage phenotypes, different T cell populations can be found in the TME of the primary tumor and metastases in PDAC patients. Owing to their different effector phenotypes, T cells essentially impact the process of tumor and metastases formation in several ways. In general, an increase of immunosuppressive (T) cells can be observed during PDAC development, while tumor directed immune functions are impaired and/or even lost [140,186,187]. Key players of the immune response against tumor cells are CD8⁺ CTL. Accordingly, several studies showed that a high tumor infiltration of CD8⁺ CTL is associated with a longer overall survival of PDAC patients [188–191].

Rahn et al. showed in human PDAC tissues that CTL are predominantly present in the TME and less in close proximity to PDAC cells [24]. One reason for this can be seen in the desmoplastic stroma containing the ECM which acts as a physical barrier for immune cells and in particular CTL [69,192]. Similar findings were reported on liver metastases. Accordingly, in a KPC mouse model small metastatic lesions in the liver show a high CD8⁺ T cell infiltration compared to large metastatic lesions. Furthermore, in small lesions CD8⁺ T cells express CD69 and no PD-1, indicating that these CTL still exhibit their effector phenotype, while in large metastatic lesions CTL express PD-1 but no CD69, indicating T cell exhaustion [193]. Importantly, this exhausted state is characterized by elevated expression of inhibitory receptors (PD-1, CTLA-4 and T cell immunoglobulin domain and mucin domain-3 (TIM-3)), decreased production of cytotoxic molecules (perforin, granzyme A/B and granzysin), decreased production of chemokines (TNF- α and IFN- γ) and higher CTL apoptosis [194,195]. For optimal CTL survival and effector function, CD4⁺ T helper cells are pivotal. In line with this finding, a high tumor infiltration of CD8⁺ CTL together with CD4⁺ T cells correlate with a better prognosis for PDAC patients [188,189]. CD4⁺ T cells are another very heterogeneous T cell population, as they can differentiate into divergent subsets (TH1, TH2, TH17 and Treg). The differentiation of TH0 helper cells into different subsets is dependent on cytokines (IL-12, IL-4, TGF β , IL-6 and IL-2) in the microenvironment [196]. The different subsets are characterized by expression of distinct cytokines and therefore show divergent impacts on all cells in the TME. Similar to CAF and macrophages, the effects can be either tumor-promoting or tumor-suppressing.

TH1 cells are regarded as tumor-suppressing as they exert various immune response activating functions e.g., they release IFN γ , which promotes recruitment of CTL, M1-macrophages and Natural Killer (NK) cells, and IL-2 which activates CTL [197,198]. On the other hand, IFN γ also induces PD-L1 expression on tumor cells, T cells, myofibroblasts and macrophages, thereby supporting CTL inhibition and immune escape in PDAC [199].

However, PDAC cells and the TME rather promote differentiation of TH2 cells which are regarded as immune-suppressing and thereby tumor-promoting [200], mainly because of the release of cytokines like IL-4, IL-5, IL-6, IL-10 and IL-13 [187]. IL-4 and IL-10 foster the differentiation of monocytes into M2-macrophages [201], IL-4 and IL-13 trigger the collagen synthesis in myofibroblasts thereby contributing to ECM remodeling [202] and

IL-13 has been shown to enhance growth of PDAC cells [203]. Accordingly, elevated levels of TH2 cytokines can be detected in plasma samples [187] and the TME of PDAC patients comprises higher numbers of TH2 cells compared to TH1 cells [204]. Moreover, high plasma levels of TH2 cytokines in patients with resectable PDAC are associated with a shorter survival [205].

Furthermore, human PDAC tissues contain higher numbers of TH17 cells compared to normal pancreatic tissue, which is associated with a shorter median survival of PDAC patients [206]. TH17 cells are regarded mainly as tumor-promoting because this T cell subset is characterized by elevated release of IL-17, IL-21 and IL-22 [197]. Importantly, IL-17 has been shown to enhance initiation and progression of PanIN in a murine PanIN model, thus being a trigger in early pancreatic tumorigenesis [207].

Another important immunosuppressive cell population in PDAC tissues are Treg. Especially in human PDAC, Tregs are mostly located in the stroma and only rarely in the epithelial layer of pancreatic ducts. Furthermore, Hiraoka et al. demonstrated a significant increase in the number of Treg during progression from low grade PanIN to an invasive PDAC. Additionally, a high prevalence of Treg in PDAC is significantly correlated with distant metastases, advanced tumor stage and high tumor grade as well as poorer prognosis [208,209]. The mechanisms by which Treg enrich in human and murine PDAC can be diverse e.g., tumor associated Treg can derive from peripheral Treg recruitment, expansion of tissue resident Treg, differentiation from local naïve T cells or conversion of conventional T cells [210]. Here, tumor- or CAF derived TGF- β can induce conversion of CD4⁺ CD25⁻ T cells into Treg [211,212]. It was also shown that Forkhead box protein 3 (FoxP3) expressing tumor cells recruit Treg by directly trans-activating CCL5 [213]. Yang et al. observed a correlation of highly expressed secreted frizzled-related protein 4 (SFRP4) and Treg infiltration in tumors of KPC mice and PDAC patients [214]. Shen et al. speculated that PDAC cell-derived sEVs induce an enrichment of human FoxP3⁺ Treg and further an overexpression of immune checkpoint molecules PD-1, PD-L1, CTLA-4 and TIM-3 as well as an enrichment of FoxP3⁺ Treg [215]. Finally, L1 cell adhesion molecule (L1CAM) expression in the pancreatic ductal epithelium was shown to promote enrichment of human Treg in PDAC by e.g., enhancing migration of Treg, decreasing proliferation of CD4⁺ effector T cells and promoting conversion into a CD4⁺CD25⁺CD69⁺ regulatory T cell phenotype [216]. Moreover, these CD4⁺CD25⁺CD69⁺ Treg are detectable at high numbers in human PDAC tissues and correlate with nodal invasion and higher grading in PDAC patients [216]. One mechanism by which Treg suppress the anti-tumor response is by interaction of CTLA-4 with CD80/CD86 on DC leading to a reduced expression of MHC class II, CD40 and CD86 molecules, all being important for Treg maturation and activation. Besides, tumor-associated DC express Indoleamine-pyrrole 2,3-dioxygenase (IDO), which suppress T cell responses and promote immune tolerance [217]. Moreover, Treg crosstalk with and are dependent on MDSCs as in a PDAC mouse model, depletion of MDSC led to a reduced recruitment and/or induction of Treg in pancreatic tumors and development/expansion of Treg seems to require a direct cell–cell interaction with MDSC [148]. Furthermore, TGF- β is not only a trigger for Treg development but also production of reactive oxygen species (ROS) by these cells contributing to oxidative stress in the TME [218].

Finally, $\gamma\delta$ T cells represent a promising tumor-suppressive T cell population because of their ability to recognize antigens in an MHC-independent manner, to present antigens to CD3⁺ $\alpha\beta$ T cells as well as their phagocytic properties. $\gamma\delta$ T cells have a prevalence of 1–10% in the blood [219]. In human PDAC tissues, $\gamma\delta$ T cells are mainly found in the tumor stroma or adjacent to or within ductal epithelium [220]. $\gamma\delta$ T cell infiltration can be promoted by CAF derived CXCL12 [221,222]. However, the presence of $\gamma\delta$ T cells is not sufficient to exert potent anti-tumor responses, because it has been shown that Galectin-3 which is expressed on PDAC cells but also $\alpha\beta$ - and $\gamma\delta$ T cells, inhibits T cell proliferation and is thus regarded as an intrinsic tumor escape mechanism [223,224]. Furthermore, kynurenine, a downstream metabolite of IDO, was identified as an inhibitor of $\gamma\delta$ T cell

cytotoxicity and proliferation in PDAC [225]. However, Daley et al. showed that $\gamma\delta$ T cells can inhibit $\alpha\beta$ T cell activation via checkpoint receptor ligation leading to suppression of CD4⁺ and CD8⁺ T cells and acquisition of an activated CD44⁺CD62L⁻ phenotype. Additionally, $\gamma\delta$ T cells were shown to decrease TNF- α expression of $\alpha\beta$ T cells in vitro. Importantly, these processes can be reversed by PD-L1 blockade suggesting that $\gamma\delta$ T cells are important modulators of a checkpoint receptor-dependent immunosuppression and pinpoint also to a tumor-suppressing role of these cells in PDAC [226].

First attempts have been undertaken to consider this T cell heterogeneity to provide a rationale for patient stratification and optimized treatment choice. Depending on the number of infiltrating lymphocytes, tumors have been categorized into immunological subtypes: T cell inflamed (“hot tumors”) or non-T cell inflamed (“cold tumors”). Owing to the low T cell infiltration especially of CD8⁺ CTL, PDAC is mostly characterized as a cold tumor [227]. Importantly, immunotherapies are often not effective in “cold” tumors explaining their common failure in PDAC [25,228].

3.6. Approaches for Targeted Therapy of T Cells

Since single agent immunotherapy with cancer vaccines or immune checkpoint inhibitors has failed so far in clinical trials [21,22,229,230], multi-agent combinations and combinations with radiotherapy have been evaluated as treatment option for PDAC. In order to boost T cell responses against cancer cells, combinations of two checkpoint inhibitors (Durvalumab and Tremelimumab) targeting the CTLA-4 and PD-1/PDL-1 axes have been evaluated [231,232]. Moreover, clinical trials were designed to assess combinations of cancer vaccines (e.g., GVAX and CRS-207) and checkpoint inhibitors or checkpoint inhibitors with oncolytic viruses (Pelareorep) or chemokine receptor inhibitors (BL-8040) based on promising preclinical data [233–236]. To date these approaches have not yielded relevant clinical improvements for PDAC patients. However, a high number of clinical trials investigating innovative immune checkpoint inhibitor-based combinations (e.g., with IL-6 antagonists (NCT04258150), agonists of stimulator of interferon genes (NCT03010176) and kinase inhibitors (NCT04820179) are underway.

As outlined above, $\gamma\delta$ T cells represent an attractive effector T cell population for cancer therapy as they recognize antigens and kill target cells MHC independently. Thus, bispecific Abs binding CD3 or V γ 9 on $\gamma\delta$ T cells and HER2/neu on PDAC cells enhanced the cytotoxicity of $\gamma\delta$ T cells via granzyme B and perforin release and led to a reduced tumor growth in a subcutaneous PDAC Xenograft model [219]. Furthermore, the tribody [(HER2)2 $\times$ CD16] activated human $\gamma\delta$ T cells and NK cells to lyse HER2 expressing PDAC cells via granzyme B release [237]. Additionally, monitoring of $\gamma\delta$ T cells subpopulations in PDAC patient’s blood and determination of their cytotoxicity can also help to optimize $\gamma\delta$ T cell-based immunotherapy [238].

Depleting Treg could also be an option to improve PDAC therapy. As Treg express CCR5, a current phase1/2 clinical trial investigates the therapeutic efficacy of CCR2 and CCR5 inhibitors in advanced PDAC with the aim to reduce Treg, MDSC and M2-polarized TAM and to increase anti-tumor immunity [209]. The bispecific antibody ATOR-1015 [CTLA-4 $\times$ OX40] induced CD8⁺ T cell activation and Treg depletion in several syngeneic tumor models including a PDAC model, thereby resulting in a tumor-specific and long-term immunological memory. Furthermore, ATOR-1015 enhanced the response to PD-1 blockade, thus providing a reasonable combination with PD-L1 inhibitors [239]. However, depletion of the entire Treg population might bear the risk for the development of autoimmunity. Additionally, preclinical studies in a PDAC mouse model demonstrated that Treg depletion does not lead to diminished immunosuppressive activity and accelerates tumor growth. Since Tregs release high amounts TGF- β , Treg depletion resulted in a reduced TGF- β levels by which in turn tumor-restraining α -SMA⁺ CAF were reprogramed into iCAFs. Furthermore, increased numbers of myeloid cells were observed in the tumors indicating that specific targeting of this particular T cell population essentially alters the

stroma cell dynamics in the TME by which unexpected tumor promoting effects might be induced [240].

In summary, PDAC-associated T cells are characterized by a great diversity exemplified by their abundance (high versus low numbers), spatial localization (in close proximity to tumor cells versus located in the TME) and effector phenotype (activated versus anergy/exhausted). Adding to this huge complexity, distinct T cell populations exert both tumor-promoting and tumor-suppressing functions. Importantly, the immunological TME composition and overall immunity in a tumor and patient, respectively, seem to be highly dependent on a variety of host intrinsic (e.g., genetics) and extrinsic factors (e.g., exposure to pathogens or environmental factors) [241] defining the above-mentioned parameters which have to be considered for an effective anti-PDAC therapy.

3.7. Heterogeneity of Endothelial Cells

In general, human PDAC tissues exhibit a high microvascular density and poorly perfused blood vessels, the latter showing a heterogeneous distribution pattern in PDAC subtypes [242,243]. Moreover, a high microvascular density along with a diminished integrity of these microvessels is associated with disease progression and poorer survival in PDAC patients [244]. This immature vasculature and the high intratumoral pressure in PDAC due to the pronounced desmoplastic stroma results in an irregular as well as disorganized tubular architecture. Murine and human studies revealed that this in turn impairs drug delivery, suppresses lymphocyte infiltration and increases hypoxia within the tumor mass [133,245–247]. In general, blood vessels support tumor growth by enabling influx of nutrients and oxygen. In addition, EC also affect tumor cells, stromal cells in the TME and therapy responses [248–250].

Tumor angiogenesis is induced by proangiogenic factors such as VEGF released by tumor and stromal cells [251]. Upon this “endothelial cell activation”, changes in the gene expression profile and phenotypes of EC occur by which these cells exert their pleiotropic effects in the tumor [252,253]. Thus, increased expression levels of Vascular Endothelial Growth Factor Receptor (VEGF-R), EGF-R and diverse cell adhesion molecules (e.g., Inter cellular Adhesion Molecule (ICAM), Vascular Cell Adhesion Molecule (VCAM), E-selectin) as well as an increased secretion of cytokines (e.g., IL-8) are observed in activated EC or tumor associated endothelial cells (TEC). These alterations essentially contribute to tumor inflammation and immune evasion in human as well as in murine tumors [254–260]. Thus, human derived material as well as murine xenografts revealed that TEC are not only morphologically distinct from their physiological counterparts, but also exhibit diverse genetic and phenotypic alterations by which these cells impact tumor development and progression as well as therapy responses. Furthermore, TEC can undergo Endothelial-Mesenchymal-Transition thereby giving rise to CAF and contributing to CAF enrichment in PDAC. In humans, this process seems to be mediated by inflammatory factors such as TNF- α [261–263].

Furthermore, Issa et al. demonstrated that upregulation of L1CAM expression on human PDAC-derived TEC increase adhesion and transmigration of PDAC cells to and through the PDAC-derived endothelium, thereby facilitating PDAC metastasis [264]. Sano et al. described that elevated expression of VCAM-1 on TEC promotes progression of PDAC as well as PanIN in a genetically engineered mouse model, but also increases the incidence of cancer-associated thrombosis in mice and patients, which is known as a poor prognostic factor in PDAC. Moreover, this is accompanied by an increased infiltration of immunosuppressive leucocytes into the tumor tissues. Blocking VCAM-1 reduces the number of thrombotic/thromboembolic events as well as infiltration of TAM and tumor-associated neutrophils and prolonged overall survival of treated mice [265]. In line with these findings, PDAC-derived TEC exhibit an enhanced expression of distinct adhesion molecules (e.g., E-selectin, ICAM-1, VCAM-1, Mucosal vascular address in cell adhesion molecule 1 (MAdCAM-1)) compared to EC from healthy pancreas. These PDAC-derived EC promote transendothelial migration of Treg thereby contributing to the immunosuppressive

TME in human as well as murine PDAC [266]. In contrast, downregulation of these adhesion molecules (e.g., ICAM-1/2, VCAM-1, MAdCAM-1, E-selectin) on TEC leads to a decreased infiltration of putative tumor-directed lymphocytes into the TME [209,267].

3.8. Approaches for Targeted Therapy of Endothelial Cells/Angiogenesis

Overall, these data indicate that TEC can exhibit different phenotypes and functions compared to their physiological counterpart, which is dependent on the tumor entity and TME composition e.g., the amount of macrophages which are a main source of VEGF. Importantly, the aberrant morphology of TEC and their altered expression profile of adhesion molecules are essential determinants for various malignancy-associated processes, such as immune evasion and drug resistance in PDAC patients [268]. Accordingly, TEC and angiogenesis, the latter being a hallmark of cancer, have been identified as promising targets for cancer therapy and many anti-angiogenic strategies have meanwhile been approved for the treatment of a variety of cancer entities. Demonstrated in preclinical and clinical studies, those strategies primarily aim at the inhibition of angiogenesis, thereby reducing nutrient supply and deactivating EC on the one hand [269–272] and normalizing the vasculature to improve drug delivery and immune cell access into the TME on the other hand [273–276]. Two commonly used anti-angiogenic drugs are Bevacizumab and Aflibercept, both binding to VEGF, and Aflibercept additionally binding to Placenta-derived Growth Factor (PGF). In addition, tyrosine kinase inhibitors (such as Sunitinib, Sorafenib, Pazopanib) that target VEGF-R2 e.g., expressed by TEC [277] are also FDA approved for anti-angiogenic cancer therapy. However, the above-mentioned strategies still fail to significantly improve overall or progression free survival of PDAC patients [278,279].

Accordingly, intensive research efforts are ongoing to identify predictive markers that allow for rational patient stratification in order to select the optimal therapeutic strategy to specifically target the aberrant vasculature in PDAC [280]. Considering the pleiotropic effects of TEC in PDAC, TEC targeting may be more effective in combination with e.g., immune checkpoint inhibitors or modified drugs (e.g., encapsulated forms) to optimize treatment strategies. Furthermore, more studies are required investigating the optimal timing for anti-angiogenetic therapies (particularly in combination with other therapies) in order to exert the maximum effect.

3.9. Heterogeneity of the Microbiome

In recent years compelling evidence has emerged that not only the TME but also the microbiome is altered in PDAC patients—in the tumor as well as in other body compartments. Experimental data support the view that the altered microbiome is not just a surrogate of the disease but rather essentially impacts development, progression and therapy responses of PDAC [11,19,20]. Miller et al. also identified an influence of the microbiome on the TME, thereby promoting PDAC development and explaining reduced therapeutic efficacy of several agents. Hence, ablation of the microbiome was shown to prevent PDAC in preclinical studies [281].

It is still not fully understood how bacteria, detectable in PDAC tissues, enter the pancreas. Potential mechanisms include the oral route or translocation from the lower gastrointestinal tract through the portal circulation or mesenteric lymph nodes [282]. Thus, it has been shown that human cystic pancreatic precursor lesions contain *Fusobacterium nucleatum* and *Granulicatella adiacens*, both bacterial populations commonly found in the oral cavity [283,284]. In line with this finding, increased amounts of *Fusobacterium* species were detected in PDAC tissues and correlated with a worse prognosis of PDAC patients [285]. Another study demonstrated that Gammaproteobacteria are the predominant bacterial species in human PDAC tissues, most of them being members of Enterobacteriaceae and Pseudomonadacea families [19].

It has been shown in patient suffering from malignant melanoma and lung cancer that the gut microbiome impacts the response to immunotherapy and chemotherapy. This finding suggests that the composition of the gut microbiome impacts activation of

the immune system [286–288] and thereby promotes cancer-associated inflammation, a mechanism which might also be relevant in PDAC. Of note, chronic pancreatitis (CP), being a risk factor for PDAC development, can be caused by microbial infections [289].

As outlined above, an inflammatory and desmoplastic environment supports various malignancy-associated processes, such as immune evasion and metastasis and impacts therapy responses [290–292], all processes related to EMT [293]. Importantly, a tumor-associated microbiome was shown to promote EMT, thereby leading to epithelial barrier alterations and tumor-promoting inflammation. In detail, it could be shown that infections by *Fuseobacterium nucleatum* lead to the loss of membranous E-cadherin [294], a key step in EMT [11,295–297]. Owing to the fact that high levels of *Fuseobacterium* are found in human pancreatic precursor lesions and PDAC tissues, it is reasonable that this might be one mechanism by which early EMT is induced in pancreatic tissues. Of note, EMT-associated alterations are already found in PDEC in human CP tissues [296] and lead to early PDEC dissemination prior to tumor formation in the pancreas in a murine mouse model [297]. Zhang et al. postulated that each microbial pathogen conquering the pancreatic tissue has the potential to induce EMT-related pathological changes [11]. Since EMT induction has been also linked to the acquisition of CSC properties and drug resistance [2,6,10], EMT/CSC induction might represent another mechanism by which the altered microbiome contributes to the pronounced therapy resistance in PDAC.

In this context, Geller et al. reported not only the presence of Gammaproteobacteria in human PDAC tissues but also that these bacteria, expressing the long form of the enzyme CDA, are able to metabolize the cytostatic drug Gemcitabine into its inactive form. These findings suggest that the presence of Gammaproteobacteria in PDAC tissues may contribute to Gemcitabine resistance in these patients [19].

Moreover, Riquelme et al. discriminated long-term survivor (LTS) PDAC patients showing a high bacterial alpha diversity from short-time survivor (STS) PDAC patients with a lower alpha diversity. They also demonstrated that the microbiome composition in PDAC tissues differs from that in normal tissues and that the gut microbiome accounts for 25% of the tumor microbiome, supporting translocation of bacteria from the gut into pancreatic tissues as one mechanism for bacterial PDAC tissue colonization [20]. Additionally, they determined greater numbers of CD3+ T cells, CD8+ T cells and granzyme B+ cells in LTS compared to STS PDAC patients, thus correlating a higher CTL infiltration with a higher microbiome diversity. These data indicate that the tumor microbiome composition influences both the extent of immune infiltration and the degree of CD8+ T cell activation in PDAC tissues [20]. It also suggests that alterations of the microbiome directly in the tumor but also in other body compartments (e.g., the gut) essentially contribute to shaping the patient's immune response. In line with these findings, it was shown in different tumor mouse models that the efficacy of the CTLA-4 inhibitor Ipilimumab depends on the presence of distinct commensal gut *Bacteroides* species (spp.) which apparently trigger TH1 immune responses, being important for the anti-tumor effects. Hence, administration of antibiotics decreased the therapeutic effect of Ipilimumab [298]. Moreover, this study also demonstrated that fecal microbiota transplantation (FMT) of human *Bacteroides* spp.-rich feces significantly increases the overall outcome of tumor bearing animals by markedly increasing the response to CTLA-4 blockade. Supporting these data, Tanoue et al. detected an 11-strain consortium of bacteria isolated from gut microbiota of human healthy control donors (HC) which can induce IFN- γ + CD8+ T cells to enhance the efficacy of immune checkpoint inhibitors in tumor-bearing mice [299]. Underscoring the therapeutic potential of FMT, a significant reduction in tumor growth was observed in PDAC bearing mice after FMT from PDAC LTS donors with no evidence of disease (LTS-NED) compared to mice obtaining FMT from STS patients or HC [20]. Moreover, tumors from mice treated with FMT of STS PDAC patients were larger than those from mice who received FMT of HC. Finally, short-term antibiotic treatment of tumor bearing mice which had received FMT from LTS-NED donors led to even larger tumors than those of untreated mice. These data suggest that certain PDAC-associated bacteria exert a tumor-promoting effect on the one

hand and that bacterial ablation by antibiotic treatment decreases the anti-tumoral efficacy of distinct bacterial species e.g., found LTS PDAC patients on the other hand.

3.10. Approaches for Microbiome Modulating Therapies

As already mentioned above, studies provide evidence that the response of cancer patients to blockade of the immune checkpoints PD-1/PD-L1 or CTLA-4 is influenced by the gut microbiome [298,299]. Tumor mouse models revealed that different microbiome compositions cause significant differences in response to treatment with PD-1/PD-L1 inhibitors [298] so that probiotics have been suggested as part of the anti-cancer therapy. Accordingly, Sivan et al. demonstrated reduced tumor growth and a beneficial response to anti-PD-1 therapy in melanoma bearing mice after oral administration of *Bifidobacterium* spp. containing probiotics. Furthermore, the superior anti-tumor effect from mice with a favorable microbiome composition could be transferred to other mice by FMT or co-housing [298,300]. In summary, these findings strongly support the therapeutic potential of FMT or certain diets/probiotics with the aim to restore the commensal (tumor-suppressing) microbiome and to displace the tumor-promoting microbiome. Combining such strategies with chemo- or immunotherapy might help to overcome therapy resistances and to improve PDAC patient prognosis.

4. Therapeutic Implications and Challenges of TME Targeting

The study by Rashid et al. revealed that consideration of different molecular subtypes e.g., those identified by [28–30], might be reasonable for stratifying PDAC patients for chemotherapy, as they provided evidence for efficacy prediction towards FOLFIRINOX treatment according to molecular tumor subtypes [32]. Adding to the multiple levels of tumor heterogeneity, stromal cell populations as well as the microbiome have been characterized by a high heterogeneity, too, implying both tumor-promoting and tumor-restraining functions. As outlined in the sections above, multiple therapeutic approaches have been developed and tested to target distinct TME components in order to improve treatment outcome of PDAC patients. However, many of these studies revealed unexpected results (e.g., by depleting the entire CAF population leading to even more aggressive tumors [131] or promising preclinical findings could not be confirmed in clinical trials [134]). Thus, until today, less than 5% PDAC patients receive a targeted therapy and significant improvements of survival are still missing [301]. Current clinical trials such as the “Precision-Panc” (NCT04161417) and the “Precision Promise” studies (NCT04229004) are ongoing with the aim to validate the therapeutic efficacy of novel individualized therapies in PDAC patients [302,303]. However, results from these trials are still pending.

The clinical failure of these strategies might also be explained by the use of inadequate preclinical models and/or an insufficient consideration of the stromal cell heterogeneity, to clearly discriminate which stromal phenotype is “friend or foe” [192,304,305]. For any kind of targeted and personalized therapy in cancer patients, pre-therapeutic samples and biopsies, respectively, are of pivotal importance and a critical determinant to assess inter- and intratumoral characteristics as well as changes during treatment courses. These allow identification of the most effective therapy and to eventually select therapies according to the profile identified [32]. In this context, surgically resected specimens provide the largest number of cells, reflect best tumor heterogeneity and enable pathologists to perform comprehensive analyses [306]. In cases of patients with an advanced disease, not being suitable for surgical resection or with recurrent tumors, fine needle aspirated (FNA) samples have been proven to be useful for identification of targets for targeted therapies. However, FNA samples only reflect a small portion of the tumor bearing the risk of not fully representing tumor and stroma heterogeneity [32,301,303]. Nonetheless, FNA-obtained samples are in certain cases the only option to obtain information on tumor and TME characteristics and help to provide individualized therapy to patients with metastatic disease whose tumor is not resectable. Liquid biopsies and analysis of cell free nucleic acids, circulating tumor cells or extracellular vesicles are additional methods to obtain information on tumor cell

characteristics. Repeated blood draws (or collection of other body fluids) are more feasible than repeated biopsy sampling and may therefore complement tissue-based analysis for longitudinal monitoring of PDAC dynamics and therapy response [307]. However, important players of PDAC biology, i.e., the TME and the microbiome are not considered in these approaches.

Of note, Birnbaum et al. already suggested to consider not only the PDAC tumor subtypes but also the stromal subtypes classified by Collisson et al., Moffitt et al. and Bailey et al. for targeted therapy in PDAC patients [28–31]. Even though targeting the TME based on stromal subtypes might be a more efficient therapeutic approach, a challenge will be to fully consider differences in distribution and extent of the TME at different sites (primary tumor versus metastases) which might be associated with divergent functional effects on PDAC development. Furthermore, the TME might be further differentially modified by chemotherapy [303,308]. Additionally, Torphy et al. identified a high stroma content as a factor for a favorable outcome in patients suffering from metastatic PDAC [309], further underscoring that the TME heterogeneity is a critical determinant for targeted stromal therapy.

The current knowledge gained from comprehensive preclinical and clinical trials demonstrating a rather limited efficacy of tumor and TME targeted therapies in PDAC patients, leads us to the following assumptions: (i) due to its extraordinary complexity and peculiarity, the TME in PDAC easily compensates pathways, interactions and effects that are modulated by mono-targeted therapies resulting in limited therapeutic efficiency, (ii) targeted therapies applied so far have impaired both tumor-promoting and tumor-restraining TME populations, thereby counteracting the beneficial therapeutic effects albeit it was intended to solely target the tumor-promoting cell populations, (iii) the functional impact of certain host-derived factors on PDAC development and on shaping therapy responses are still insufficiently understood and considered (e.g., the host microbiome) and (iv) the usage of reliable biomarker (not only in tumor tissue but also other compartments) for therapy decision making has still to be improved for treatment of this tumor entity.

Accordingly, concepts aiming at restoration and normalization of a physiological, tumor-restraining microenvironment, respectively, rather than at precisely targeting distinct TME components might be more effective strategies [180,310]. For example, Kocher et al. focused on the stroma in patients with locally advanced or metastatic PDAC and replenished the Gemcitabine-Nab-Paclitaxel chemotherapy with ATRA in a phase I clinical trial. Clinical safe and tolerable efficiency and successful reprogramming of the TME was detectable [311] which was also observed in other preclinical studies [137]. Another “normalization” or “reprogramming” strategy might be the modulation of the patient’s microbiome. As outlined above, the microbiome substantially influences the efficacy of chemotherapeutic drugs in PDAC-patients [11,19,20] as well as the response to immune checkpoint inhibition [298]. Additionally, restoration of a tumor-restraining microbiome by application of distinct microbiome species, e.g., such as FMT, may not only overcome drug resistances, but also help to boost the patient’s immune system to induce a long-term control of the tumor burden [11,20].

Overall, the above-mentioned studies and continuously elaborated novel concepts have essentially broadened our understanding of this challenging disease. However, additional studies are needed to further dissect the dynamics and heterogeneity of the TME regarding the tumor-promoting and tumor-restraining functions in order to translate this knowledge into personalized therapeutic concepts to improve the prognosis of PDAC patients. Consideration of this heterogeneity and its impact on PDAC development and therapy responses is enabled by collecting and comprehensively analyzing tumor, stroma and the patient’s microbiome to generate an integrated tumor subtype. This identified subtype allows stratification of each patient for the individualized and most effective therapy (Figure 2).

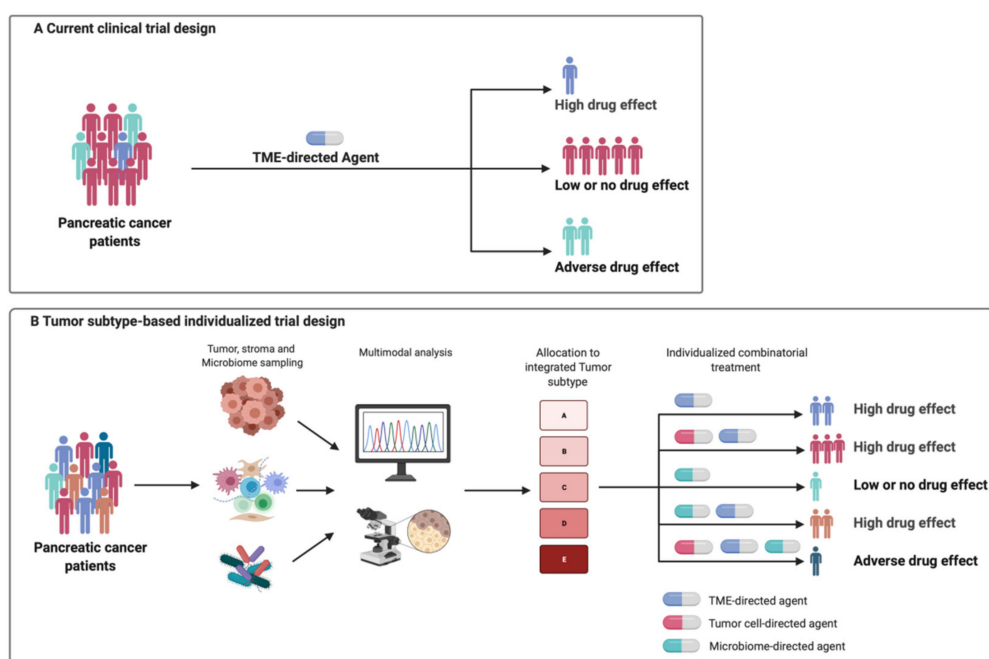


Figure 2. Current and future therapeutic concepts for the treatment of pancreatic cancer patients. **(A)** In current clinical trials, pancreatic cancer patients are not stratified according to distinct tumor and stroma characteristics. Accordingly, the given therapy exerts an anti-tumor effect only in few patients. However, in the majority of patients this strategy fails or has even adverse effects. **(B)** A tumor subtype-based individualized trial design implies sampling of the patient's tumor, stroma and microbiome prior to treatment and multimodal analysis of these samples will generate an integrated tumor subtype for each patient. Considering these tumor, stroma and microbiome characteristics, the identified subtype allows stratification of pancreatic cancer patients for the treatment strategy with the best predicted outcome. Figure created with [BioRender.com](https://www.biorender.com/).

5. Conclusions

Extensive preclinical and clinical evaluation of PDAC has resulted in detailed characterization of its complexity on multiple levels. In this article, we summarized how PDAC biology is determined by a plethora of cell types and microbiota shaping the TME and their context-dependent pro- or anti-tumorigenic characteristics. This challenging level of complexity is further increased by countless interactions between the involved cell types. In the future, increased efforts will be needed to translate these insights into TME heterogeneity into clinically meaningful benefits for PDAC patients. To this end, we propose comprehensive analysis of patient material (tumor, tumor stroma and microbiome) and the development of integrated tumor subtypes based on this data base. Respective integrated tumor subtypes may then serve for informed patient stratification in clinical trials and truly individualized therapeutic decisions.

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Abbreviations

ADEX: aberrantly differentiated endocrine exocrine; α -SMA: Alpha-smooth muscle actin; APC: Antigen presenting cells; apCAF: antigen presenting carcinoma associated fibroblasts; ATRA: All-trans retinoic acid; CCL: Chemokine (C-C motif) ligand; CAF: Carcinoma associated fibroblasts; CD: Cluster of differentiation; CDA: Cytidine deaminase; CP: Chronic pancreatitis; CSC: Cancer stem cells; CTLA-4: Cytotoxic T-Lymphocyte-Associated Protein 4; CXCL: chemokine (C-X-C motif) ligand; CTL: Cytotoxic T cells; DC: Dendritic cells; EC: endothelial cells; ECM: Extracellular matrix; EGF: Epidermal Growth Factor; EGFR: Epidermal Growth Factor-Receptor EMT: Epithelial-Mesenchymal-Transition; FNA: Fine needle aspirated; FMT: Fecal microbiota transplantation; FoxP3: Forkhead box protein 3; G-CSF: Granulocyte-Colony Stimulating Factor; HC: Healthy control donor; HGF: Hepatocyte Growth Factor, HIF-1: Hypoxia-inducible factor-1; HSC: Hepatic stellate cells; HMF: Hepatic myofibroblasts; iCAF: inflammatory CAF; ICAM: Intracellular Cell Adhesion Protein; IDO: Indoleamine-pyrrole 2,3-dioxygenase; IL: Interleukin; IFN- γ : Interferon-gamma; IPMN: Intraductal papillary mucinous neoplasm; JAK: Januskinase; L1CAM: L1 cell adhesion molecule; LPS: Lipopolysaccharide; LTS: Long-term survivor; PanIN: Pancreatic Intraepithelial Neoplasia; MAdCAM-1: Mucosal vascular addressin cell adhesion molecule 1; M-CSF: Macrophage-Colony Stimulating Factor MDSC: Myeloid-derived suppressor cell; MHC: Major histocompatibility complex; myCAF: myofibroblastic CAF; NED: No evidence of disease; NK: Natural killer; PDAC: Pancreatic ductal adenocarcinoma; PDEC: Pancreatic ductal epithelial cells; PD-1: Programmed cell death protein 1; PDGF: Platelet-Derived Growth Factor; PD-L1: Programmed cell death 1 ligand 1; PI3K: Phosphatidylinositol 3-kinase; PGF: Placenta-derived Growth Factor; PMN: polymorphonuclear; PSC: Pancreatic stellate cells; ROS: Reactive oxygen species; sEV: small extracellular vesicle; SFRP4: secreted frizzled-related protein 4; Shh: Sonic hedgehog; SMO: Single transducing component smoothened; STAT: Signal transducer and activator of transcription protein; STS: Short-term survivor; TGF- β : Transforming Growth Factor-beta; TAM: Tumor-associated macrophages; TEC: Tumor associated Endothelial Cells, TIM-3: T cell immunoglobulin domain and mucin domain-3; TME: Tumor microenvironment; TNF- α : Tumor Necrosis Factor-alpha; Treg: Regulatory T cells; VCAM: Vascular Cell Adhesion Molecule; VEGF: Vascular Endothelial Growth Factor, VEGF-R: Vascular Endothelial Growth Factor-Receptor.

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Epithelial and mesenchymal pancreatic cancer cells exhibit different stem cell phenotypes being associated with different metastatic propensity

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Abstract (350 Wörter für Journal of Experimental & Clinical Cancer Research)

Background: Pancreatic ductal adenocarcinoma (PDAC) is the most common malignant pancreatic tumor in western countries, characterized by a low 5-year survival rate of only 11 %. Owing to a lack of early and specific symptoms, most patients are diagnosed at advanced or even metastasized stages essentially impairing treatment efficacy. Metastatic outgrowth requires a high plasticity of tumor cells implying phenotypic switching in response to changing environments. One important process leading to phenotypic switching is the Epithelial-Mesenchymal-Transition (EMT) and its reversion, the Mesenchymal-Epithelial-Transition (MET). This process has been associated with the gain of cancer stem cell (CSC) properties being regarded as drivers of tumor initiation at primary and secondary sites. Still, it is poorly understood whether different CSC phenotypes exist along the EMT/MET axis and how these impact malignancy associated properties. Thus, this study aimed to characterize CSC populations of epithelial and mesenchymal PDAC cells and how these relate to functional phenotypes. **Methods:** For this purpose, single-cell-cloning of the two PDAC cell lines Panc1 and Panc89 was performed to isolate and expand CSC- (Holoclone cells) and non-CSC (Paraclone cells) clones. Cell variants were characterized regarding cell growth, migration, invasion, cell adhesion and tumorigenicity.

Results: Panc1 Holoclone cells showed a mesenchymal phenotype dominated by high expression of the stemness marker Nestin, while Panc89 Holoclone cells exhibited an epithelial phenotype with elevated expression of the stemness marker SOX2. Moreover, Panc89 Holoclone cells showed enhanced cell growth and colony formation ability compared to Panc1 Holoclone cells. In contrast, slowly proliferating Panc1 Holoclone cells were highly invasive, while Panc89 Holoclone cells showed a slow cluster-like invasion. In line with these findings, a higher number of metastases was formed by Panc1 Holoclone cells *in vivo*. However, Panc89 Holoclone cells led to formation of larger tumors limiting survival of inoculated mice. Moreover, epithelial and mesenchymal CSC-clones showed clear differences with respect to organ manifestation.

Conclusion: Overall, these data support the view of different CSC phenotypes in PDAC cells that are linked to distinct EMT/MET phenotypes and that manifest in a different metastatic propensity.

Background

Pancreatic ductal adenocarcinoma (PDAC) is the most common malignant pancreatic tumor, characterized by a still poor prognosis with a 5-year survival rate of only around 12% and an still ongoing increase in death rate (1,2). Due to the lack of early and specific symptoms, the majority of patients are diagnosed at an advanced or even metastasized stage leaving only palliative treatment. The only curative option is surgery which, however, is eligible to about 20% of patients (3,4). PDAC metastasizes predominantly in the liver, but also in lung and peritoneum (4–6). A significantly shorter disease-free survival and overall poorer prognosis are described for resected PDAC patients who developed liver and peritoneal metastases in contrast to patients with solitary lung metastases (7).

The multistep process of metastasis starts with the dissemination of tumor cells from the primary tumor and ends with proliferation and outgrowth of visible metastases at secondary sites (8–10). As a prerequisite for dissemination, carcinoma cells have to acquire a motile and invasive phenotype, which is commonly described to be achieved by Epithelial-Mesenchymal-Transition (EMT) (11,12). EMT is associated with the downregulation of adhesion molecules and loss of epithelial proteins like E-cadherin and conversely, a gain of mesenchymal characteristics like increased expression of mesenchymal markers such as Vimentin, L1CAM or the transcription factors ZEB1, ZEB2 and OVOL2 (11–17). Furthermore, this loss of cell differentiation of carcinoma cells has been associated with the acquisition of cancer stem cell (CSC)-properties (18–20). Thus, Mani et al. (18) were the first who demonstrated that breast cancer cells that have undergone EMT acquire a stem cell-like phenotype, and likewise, stem cell-like cells resemble cells that have undergone EMT.

CSCs are a small group of cancer cells within a cancer cell population with the unique ability to both self-renewal and generation of more differentiated cells. Owing to these unique features, CSCs are regarded as essential for tumor initiation at primary and secondary sites, including PDAC (21–23). Recent studies indicate that CSC-properties can be gained and lost depending on the microenvironment (20,24–27), indicating that CSCs are not a stable, but highly plastic cell population. Several markers, e.g. ABCG2, CD133, CD24, CD44, Nanog, Nestin and SOX2, have been proposed for identification of CSCs in PDAC already indicating a high heterogeneity within the CSC population (28–34). The intermediate filament Nestin and the stem cell (SC)-transcription factor SOX2 seem to play a role in the maintenance of CSC-properties (35,36). Nestin impacts cell motility and EMT-properties in PDAC cells and it has been shown

that knockdown of Nestin in PDAC cells reduces tumor incidence and volume, as well as formation of liver metastases in a murine PDAC model (37,38). Elevated SOX2 expression has been rarely detected in pancreatic intraepithelial neoplasia rather than poorly differentiated tumors and neurally invasive tumors supporting a role of this factor in later stages of tumorigenesis and metastasis(39). In line with these findings it has been shown that SOX2 is involved in Mesenchymal-Epithelial-Transition (MET), the reversion of EMT, as knockdown of SOX2 in colorectal cancer (CRC) cells altered expression of key genes involved in the EMT process including E-cadherin and Vimentin (40). Furthermore, *de novo* SOX2 expression in pancreatic cancer cells is sufficient to promote self-renewal, de-differentiation and impacting stemness characteristics *via* modulating specific cell cycle regulatory genes and EMT-driver genes (32). *In vitro*, self-renewal abilities are assessed by colony formation and here, different colony types exhibiting distinct morphologies and different CSC potential have been described (41). While Holoclones are considered to be the colony type with the highest amount of CSCs, Paraclones are characterized by more differentiated cells (41,42). Meroclones form an intermediate stage of these two colony types and differ from Holoclones mainly in their lower proportion of CSCs (41,42). Our group already demonstrated that Panc1 Holoclone cells derived by single cell cloning from the parental PDAC cell line Panc1 showed elevated and exclusive expression of the CSC-marker Nestin along with a more pronounced mesenchymal phenotype compared to Paraclones (41). Of note, experimental evidence has been provided that also carcinoma cells with an epithelial phenotype can contribute to metastasis, using a cluster-like migration pattern, which has also been described for PDAC cells (43). Moreover, recent studies indicate that so called hybrid cells concomitantly showing epithelial and mesenchymal characteristics exhibit the highest plasticity regarding cancer stemness, tumor initiation capacity as well as adaptation capability so that these cells are regarded as the cells with the highest probability to metastasize (43). Still, it is poorly understood whether different CSC phenotypes exist along the EMT/MET axis and how these impact malignancy associated properties. Thus, to explore whether and how different EMT states are associated with CSC-properties in PDAC and how this impacts tumor cell growth and metastasis, Holo- and Paraclone cells were isolated and expanded from another PDAC cell line (Panc89). In contrast to mesenchymal Panc1 cells which have presumably already undergone EMT but still are derived from a primary PDAC, Panc89 cells originate from a lymph node metastasis and have presumably undergone EMT and MET during the metastatic process.

Using this well-defined cell model the present study aimed at a comprehensive *in vitro* and *in vivo* analysis of isolated Holo- and Paraclone cells from Panc1 and Panc89 cells with respect to EMT- and CSC-properties as well as tumorigenicity and metastasis. Overall, our data support the view of great plasticity and heterogeneity within cancer (stem) cells in PDAC, differentially impacting tumor growth and metastatic behavior.

Methods

Cell lines and cell culture

As a model for epithelial PDAC cells, the human cell line Panc89 (kindly provided by Prof. T. Okabe, University of Tokyo, JP) originating from a lymph node metastasis of PDAC was used. As a model for mesenchymal PDAC cells, the human cell line Panc1 was used (purchased from ATCC, Manassas, Virginia US) originating from a primary tumor of a PDAC patient. Holo- and Paraclone cells of both cell lines were isolated and expanded as outlined in chapter 2.2. All cell lines were cultivated in Panc-medium (RPMI 1640 supplemented with 10% FCS, 1% L-Glutamine and 1% sodium pyruvate (Biochrom, Berlin, DE)).

For analysis of PDAC cell adhesion behavior to endothelial cells of different metastatic sites, lung (HuLEC-5a) and liver (TMNK-1) endothelial cell lines as well as the mesothelial cell line Met-5a were used. HuLEC-5a cells (purchased from ATCC, Manassas, Virginia US) are human microvascular endothelial cells that are derived from the lungs of a male patient ⁴⁵and were cultured in MCDB 131 (Biochrom, Berlin, DE) medium containing 10% FCS, 1% L-Glutamine, 10 ng/ml Epithelial Growth Factor and 1 µg/ml hydrocortisone. TMNK-1 (purchased from NIBI-OHN JCRB cell bank, Osaka, JP) are human liver sinusoidal endothelial cells(46) and originate from the liver of an healthy female patient (46) and were cultured in DMEM (Biochrom, Berlin, DE) containing 10% FCS, 1% L-Glutamine and 1% Penicillin-Streptomycin. Met-5a cells (purchased from ATCC, Manassas, Virginia US) originating from mesothelium, were isolated from the pleural fluids of non-cancerous individuals (47,48) and cultured in Medium199 (Sigma Aldrich) containing 10% FCS, 400nM hydrocortisone, 870nM porcine insulin and 20nM HEPES.

Single cell cloning and clone expansion

Single cell cloning and expansion was performed with PDAC cell lines to isolate and expand single cell derived Holo- and Paraclone cells from the parental cell population. Single cell cloning of Panc1 cells to isolate Panc1 Holo- and Paraclone cells, respectively, was already performed previously (Knaack et. al., 2018). To isolate Holo- and Paraclone cells from Panc89 cells, the parental cells were pre-diluted to a cell count of 1000 cells per ml Panc-medium. From this cell suspension, 100 µl (corresponding to 100 cells) were added to 20 ml of Panc-medium to obtain a concentration of one cell per 200 µl of cell medium, which were filled in each well of a flat 96-well plate. The plate was centrifuged and the first screening was performed directly after centrifugation by imaging the plate in the brightfield channel of a

NYONE® Scientific Imager (SynenTec GmbH, Elmshorn, DE) and with the brightfield channel of EVOS microscope (AMG, Bothell, US). Only wells containing exactly one cell were considered for further monitoring and expansion. The microscopical screening was performed twice a week. After 12-20 days, the colony shape was determined and the colonies monitored until a cell confluence of about 80%. Cell clones with a stable colony morphology were detached from one well and transferred to one well of a 6-well plate. The morphology of expanded cell clones was continued to be checked regularly. Only clones with a clear CSC- or non-CSC morphology and stable phenotype were further cultivated, expanded and used for phenotypic and functional characterization. Phenotypes were regularly checked by marker analysis *via* qPCR.

Colony formation assay (CFA)

Colony formation ability was analyzed by CFA. For this purpose, 400 cells were seeded as duplicates or triplicates in 6-well plates in Panc-medium. After cultivation for 8-11 days, colonies were fixed with 4% Paraformaldehyde (Thermo Scientific, Schwerte, DE) for 10 min, stained with 0.1% crystal violet (Merck Millipore, Darmstadt, DE) for 1 h, washed in dest. water and air-dried at room temperature. Only colonies containing more than 50 cells were counted and their morphology regarding Holo- and Paraclone cells was determined. Holoclones consisted of tightly, homogenously clustered cells with a regular borderline, while Paraclones consisted of dispersed, larger cells with an irregular boundary (49). Meroclones defined by an intermediate colony type (49) were neglected in this study.

RNA isolation and RT-qPCR

Total RNA was isolated using the total RNA kit peqGOLD (PeqLab, Erlangen, DE) and subjected to reverse transcription according to manufacturer's instructions (Fermentas *via* Thermo Fisher Scientific, Darmstadt, DE). The qPCR analysis was performed in duplicates on a Light-Cycler 480 (Roche, Basel, CH) for a maximum of 50-60 cycles ending with a melting curve analysis for primer quality control. Used primers (Eurofins, Ebersberg DE; RealTime Primers *via* Biomol, Hamburg, DE), primer sequences and annealing-temperatures are listed in Table 1. For relative quantification of RNA levels, C_T-values of genes of interest were normalized to the respective C_T-value for the reference gene GAPDH.

Table 1: Target genes, primer sequences and annealing temperatures used for quantitative polymerase chain reaction

Target	5` - 3` Sequence	Annealing [C°]
<i>CDH1</i> (E-cadherin)^{**}	fw - TGCTCTTGCTGTTTCTTCGG rv - TGCCCCATTTCGTTCAAGTAG	55
<i>GAPDH</i>[*]	fw - TCCATGACAACCTTTGGTATCGTGG rv - GACGCCTGCTTCACCACCTTCT	58
<i>L1CAM</i>^{**}	fw - GAACTGGATGTGGTGGAGAG rv - GAGGGTGGTAGAGGTCTGGT	58
<i>NES</i> (Nestin)[*]	fw - GAAACAGCCATAGAGGGCAAA rv - TGGTTTTCCAGAGTCTTCAGTGA	58
<i>OVOL2</i> (ZNF339)^{**}	fw - GGGACAAGCTCTACGTCTGC rv - GTCTGTCTCTCCCTTCCTTC	58
<i>SOX2</i>[*]	fw - TCCCATCACCCACAGCAAATGA rv - TTTCTTGTCGGCATCGCGGTTT	58
<i>VIM</i> (Vimentin)^{**}	fw - TCCAAGTTTGCTGACCTCTC rv - TCAACGGCAAAGTTCTCTTC	58
<i>ZEB1</i>[*]	fw - TCCATGCTTAAGAGCGCTAGCT rv - ACCGTAGTTGAGTAGGTGTATGCCA	61
<i>ZEB2</i>^{**}	fw - CACATCAGCAGCAAGAAATG rv - AAACCCGTGTGTAGCCATAA	58

* = Primers purchased from Eurofins Genomics GmbH (Ebersberg, DE). Stocks of forward (fw) and reverse (rv) diluted at 1 pmol/μl in nuclease-free ddH₂O

** = Primers purchased from RealTime Primers (via Biomol, Hamburg, DE). Provided as stocks of 50 μM primer mix (containing fw and rv primer) in nuclease-free ddH₂O supplemented with 10 mM Tris-HCL and 0.1 mM EDTA (pH 7.5)

Analysis of CSC- and EMT-marker *via* immunofluorescent staining

For the analysis of different CSC- and EMT-marker on protein level in Panc1 and Panc89 parental, Holo- and Paraclone cells, an immunofluorescent staining (IFS) was performed. Based on RNA analysis data, all Panc1 cell variants were stained with anti-NESTIN (clone 10C2; Thermo Scientific, Schwerte, DE) and anti-ZEB2 (polyclonal; Novus Biologicals, Wiesbaden Nordenstadt, DE) antibodies, while all Panc89 cell lines were stained with anti-SOX2 (clone D6D9; Cell Signaling Technology, Danvers, MA - US) and anti-L1CAM (clone UJ127.11; Gerd Moldenhauer, German Cancer Research Center, Heidelberg, DE) antibodies.

First, glass coverslips were placed in each well of a 12-well plate. Afterwards, 0.5×10^4 cells/well were seeded in each well. After 48 h, cell culture medium was removed and the cells were washed with PBS. Fixation of the cells was performed by incubation with 4% PFA for 15 min at RT. Next, the coverslips were washed 3×5 min with PBS and incubated with ice cold methanol (MetOH) for 10 min at -20°C for permeabilization of the cells. Afterwards, the coverslips were washed with PBS and cells were blocked with 4% bovine serum albumin (BSA)-0.3% TritonX-100 in PBS for 1 h at RT. After a washing step with 0.3% TritonX-100/PBS, incubation with the primary antibodies was performed.

Concomitant double IFS staining of NESTIN and ZEB2 in Panc1 cells:

Incubation of the antibodies and Hoechst 33258 (Merck Millipore, Darmstadt, DE) was carried out in 1% BSA-0.3% TritonX-100/PBS and in a humidity chamber. Incubation of the primary antibodies was performed at 4°C overnight (ON). Anti-Nestin antibody (Thermo Scientific, Schwerte, DE) was diluted 1:200 (= 5 µg/ml), anti-ZEB2 antibody (Novus Biologicals, Wiesbaden Nordenstadt, DE) was diluted 1:50 (= 2 µg/ml), mouse IgG1 isotype control (R&D Systems GmbH, Wiesbaden, DE) was diluted 1:100 (= 5 µg/ml) and rabbit IgG isotype control (Bio-Techne, Minnesota, US) was diluted 1:500 (= 2 µg/ml). Incubation of primary antibodies was performed as a concomitant double staining incubating with both primary antibodies at the same time. After ON incubation, cells were washed with 0.3% TritonX-100/PBS. Afterwards, anti-mouse antibodies conjugated with AlexaFluor 488 (Invitrogen, Carlsbad, US), anti-rabbit antibodies conjugated with AlexaFluor 647 (Invitrogen, Carlsbad, US) and Hoechst 33258 were each diluted 1:500 in 1% BSA-0.3% TritonX-100/PBS and added to the cells for 1h at RT. After washing with PBS and dH₂O, coverslips were sealed with Fluor Safe Reagent (Electron Microscopy Sciences, Hatfield, PA) and transparent nail polish.

Sequential IFS staining of SOX2 and L1CAM in Panc89:

Incubation of anti-SOX2 (Cell Signaling Technology, Danvers, MA - US) and related secondary antibody were carried out in 1% BSA-0.3% TritonX-100/PBS and in a humidity chamber. Incubation of anti-L1CAM and related secondary antibody, as well as Hoechst 33258 staining were carried out in 1%BSA/PBS. Anti-SOX2 antibody was diluted 1:200 (= 25 µg/ml), anti-L1CAM antibody was diluted 1:100 (= 10 µg/ml), mouse IgG1 isotype control was diluted 1:50 (= 10 µg/ml) and rabbit IgG isotype control was diluted 1:40 (= 25 µg/ml). Incubation of primary antibodies was performed sequentially, starting with the staining of SOX2 at 4°C and ON. After incubation with anti-SOX2 antibody, cells were washed with 0.3% TritonX-100/ PBS and incubation with anti-rabbit antibody conjugated with AlexaFluor 647 diluted 1:500 in 1% BSA-0.3% TritonX-100/PBS was performed for 1h at RT. After washing with PBS, incubation with anti-L1CAM antibody was carried out for 1h at RT. After washing with PBS, cells were incubated with the anti-mouse antibody conjugated with AlexaFluor 488 and Hoechst 33258, both diluted 1:500 in 1% BSA/PBS for 1h at RT. After washing with PBS and dH₂O, coverslips were sealed with Fluor Safe Reagent and transparent nail polish. Image acquisition and staining evaluation of all cell lines were performed with the Lionheart FX Automated Microscope and related software (Gen5 Data Analysis Software (BioTek, Bad Friedrichshall, DE).

RNA sequencing and transcriptomic analysis

Total high-purity RNA of all cell variants was isolated using the total RNA kit peqGOLD (PeqLab, Erlangen, DE) with an additional DNase digestion step according to manufacturer's specifications. Afterwards, RNA-triplicates (3 biological replicates) of each cell line were placed in a 96-well plate in a concentration of at least 150 ng/15 µl RNase-free water. RNA sequencing was performed at the Institute of Clinical Molecular Biology (Kiel, DE) under supervision of Sören Franzenburg. Input total RNA was quantified using the Quant-it RNA Assay (Thermo Fisher, Waltham, USA) and RIN scores were determined using the Agilent Tape Station (Agilent, Santa Clara, USA). All RIN scores were > 8. 200 ng input total RNA were processed using the Illumina stranded mRNA kit (Illumina, San Diego, USA). Libraries were sequenced on the Illumina NovaSeq 6000 SP Flowcell using 100bp paired-end reads. Transcriptomic analysis of mRNA sequencing data was performed by Hauke Busch (Medical Systems Biology Group, Institute of Experimental Dermatology, University of Lübeck, DE). Raw sequencing data (fastq format) was mapped against the human transcriptome (Ensembl GRCh38.106) using kallisto (v0.46.1) (50), and gene expression was summarized from the scaledTPM values of the transcripts using tximport (51). Differential gene expression was performed using DESeq2 (52). Gene set

enrichment analysis (GSEA) on the shrunken log₂ fold changes from apegglm (53) was performed using GAGE (v2.48.0) (54) against HALLMARK, REACTOME and Gene Ontology Biological Processes (GOBP) gene sets extracted from the msigdb R package (v7.5.1).

Cell growth analysis

Parental Panc1 and Panc89 cells as well as their derived Holo- and Paraclone cells were seeded at 5×10^3 cells/well in triplicates in 96-well plates for seven days (168 h). The cells were imaged every day with the brightfield channel (confluence operator) and the UV channel (nuclei count operator) of the NYONE® Scientific Imager (SynenTec GmbH, Elmshorn, DE). To analyze the number of nuclei, every day three wells per each cell variant were dyed with Hoechst33342 (1:5000; Thermo Fisher, Carlsbad/California). The examined data were analyzed via nonlinear regression (curve fit) to maintain the growth rate (κ) for logistic growth for the cell confluence analysis ($k_{\text{CELL CONFLUENCE [\%]}}$) and the nuclei count analysis ($k_{\text{NUCLEI COUNT [\#]}}$) (YT®-Software (SYNEN-TEC GmbH, Elmshorn, DE) in GraphPad Prism 9.5.0 (GraphPad Software, California, US).

Migration assay

To analyze cell migration of Panc1 and Panc89 cell variants, two-chambered silicone inserts by Ibidi® (Ibidi GmbH, Martinsried, DE) with a defined cell-free gap were used. Before use, inserts were pre-warmed at 37°C for about 30 min and then placed into wells of a 24-well plate. Per each chamber of an insert, 5×10^4 PDAC cells were seeded in a volume of 75 - 100 μ l Panc-medium and incubated for minimum 24 h or to a cell confluence of approximately 100%. Afterwards, each well was filled with 1 ml of PBS to remove the inserts, resulting in a cell-free gap between the cell layer of both chambers. The PBS was removed and the wells were refilled with 1 ml of FCS-free cell Panc-medium.

Migration behavior was determined by measuring cell confluence on the cell-free gap over a period of 32h using the CELLAVISTA®/NYONE® Imager and the YT® *Wound healing*-operator (SynenTec GmbH, Elmshorn, DE). Measuring intervals were 8 h and the plate was stored in the incubator at 37°C, 5% CO₂ and 85% relative humidity during the time between measurements. Migration rate was determined by measuring the cell confluence within the gap. Initially, the operator measured opposing areas at the edge of the gap and reported the cell confluence at this starting point as being between 10-20%. Throughout the experiment, the cell confluence was evaluated to this initial value, with an increase in cell confluence within the gap defined

as migration. If no migration occurred, the cell confluence within the gap remained unchanged.

Invasion assay

Invasive behavior of Panc1 and Panc89 cell variants was analyzed in spheroid invasion assays. For this purpose, 5×10^3 cells/well were seeded in 96-well ultra-low-attachment plates (Corning, Kennebunk ME, US; faCellitate, Mannheim, DE) in 200 μ l Panc-medium enabling spheroid formation. Directly after seeding, the plate was centrifuged. After spheroid formation for 48 h, 150 μ l of cell Panc-medium was removed from each well and replaced by 50 μ l Matrigel (Corning, New York, United States) to achieve a protein concentration of 6.25 mg/l. After addition of Matrigel, the plate was centrifuged and the plate was incubated at 37°C, 5% CO₂ and 85%. After 30 min, the first imaging was performed using NYONE® Scientific imaging device and the YT® *Spheroid invasion*-operator. Duration for monitoring cell invasion was chosen according to invasive behavior of the PDAC cell variants. To account for these differences, Panc1 cell variants were imaged at time point 0, 24, 48, 72h, while Panc89 cell variants were measured at time point 0h as well as after 5 and 7 days. The determination of invasive fronts was carried out by visual inspection of the image data generated by the NYONE® Scientific Imager (YT®-Software, SynenTec GmbH, Elmshorn, DE) and by determining the number of protruding cells or cell clusters in each spheroid. The invasive distance was measured using a digital measuring instrument integrated into the YT®-Software. Specifically, the invasive distance was determined by utilizing a green-marked circle corresponding to the spheroid diameter as the starting point, while the distal edge of the cells facing away from the spheroid was designated as the end point of maximum invasion.

Adhesion assay

To analyze cell adhesion of Panc1 and Panc89 cell variants, 1×10^5 /well HuLEC-5a, Met-5a or TMNK-1 cells, each diluted in 200 μ l of their respective medium, were seeded in a 96-well plate. After 24h, parental Panc1 and Panc89 or their derived Holo- and Paraclone cells were added. Before, 5×10^5 cells/ml PDAC cells were suspended in FCS-free Panc-medium and stained with CellTracker Green CMFDA (5-chloromethylfluorescein diacetate, diluted 1:2000) (Thermo Scientific, Schwerte, DE) for 30 min at 37° C under consistent agitation.

After staining, the cells were washed with Panc-medium and 1×10^4 /well PDAC cells were seeded in 200 μ l Panc-medium onto HuLEC-5a, Met-5a or TMNK-1 cells. Afterwards, the plate

was centrifuged at 300×g for 5 min to ensure subsequent contact of all PDAC cells to endothelial or mesothelial cells. Immediately afterwards, the plate was measured in the NYONE® Imager (SynenTec GmbH, Elmshorn, DE), determining maximal fluorescence count of PDAC cells ($t=0$ h). To determine fluorescence of PDAC cells adhered to the endothelial or mesenchymal cells, measurement was performed after 4h. Before measuring, medium containing non-adhered PDAC cells was removed from the wells and replaced with fresh Panc-medium. The number of fluorescent cells at 4 hours after initial measurement was compared to the number of fluorescent cells at time 0 h to determine the adhesion rate. Adhesion rate was then calculated as percentage of adherent cells by dividing the number of fluorescent cells at each time point by the number of fluorescent cells at time 0 h.

Tumorigenicity assay *in vivo*

All animal studies were executed in compliance with European guidelines for care and use of laboratory animals and approved by local authorities (V242-44598/2018 (80-8/18)). The tumorigenicity assay for Panc1 Holo- and Paraclone cells was already performed (41). The analysis of tumorigenicity and metastatic behavior of Panc89 Holo- and Paraclone cells was performed accordingly(41). For this purpose, 1×10^4 cells of either Panc89 Holo- or Paraclone cells were diluted in 75 μ l PBS and intrasplenically inoculated in 8-week-old, female SCID-beige mice (each group $n=10$) (Charles River, Sulzfeld, DE). Progression of tumor formation was monitored regularly by palpation and abdominal ultrasound examination using Vevo 770 (FUJIFILM VisualSonics Inc., Toronto, CA). Mice were sacrificed depending on tumor burden and general health condition of the mice. Afterwards, organs and formed tumors were fixed in 4% PBS-buffered formalin and embedded in Paraffin for sectioning and immunohistological examination.

Immunohistochemical staining of Paraffin-embedded tissue sections

Formalin-fixed and Paraffin-embedded (FFPE) organs and tumors from Panc1 or Panc89 Holo- or Paraclone inoculated mice were used for immunohistochemical (IHC) analysis. Antigen retrieval was performed as listed in Table 2 and tissue sections were stained with antibodies listed in Table 3. Incubation of anti-PanCK, anti-L1CAM, anti-ZEB1, anti-NESTIN and anti-SOX2 was performed 1h at RT (30 min for anti-E-cadherin), followed by 30 min incubation at RT for all secondary Abs (15 min for ZEB2 secondary antibody).

Table 2 List of antigen retrieval conditions

Antigen	Buffer (pH)	Temperature, Time
anti-E-Cadherin	S1699 (pH 6.1)	Steaming 121°C, 10 min
anti-L1CAM	EDTA (pH 8.0)	1. microwave broiling 800 watts 2. microwave 560 watts, 3×5 min
anti-Nestin	Citrate buffer (pH 6.0)	Steaming 121°C, 10 min
anti-PanCK	Target Retrieval Solution, Citrate (pH 6.1, 10x), S1699 (DAKO Agilent, Santa Clara, US)	1. Boiling 2. Waterbath, 95°C, 20 min
anti-SOX2	Target Retrieval Solution, Citrate (pH 6.1, 10x), S1699 (DAKO Agilent, Santa Clara, US)	Steaming 125°C, 4 min
anti-Vimentin	Target Retrieval Solution, Tris/EDTA (pH 9.0, 10x), S2367 (DAKO Agilent, Santa Clara, US)	Waterbath 85°C, 20 min
anti-ZEB1	Citrate buffer (pH 6.0)	Steaming 121°C, 10 min
anti-ZEB2	Citrate buffer (pH 6.0)	Steaming 121°C, 10 min

Table 3 List of antibodies used for IHC of FFPE tissue sections from Panc1 or Panc89 Holo- or Paraclone cell inoculated animals

Primary antibody	Secondary antibody	Isotype
Pan-Cytokeratin (PanCK) (12,7 µg/ml; clone AE1/AE3; DAKO Agilent, Santa Clara, US)	Goat anti-mouse biotin (1:200; LS-C149505; LS-Bio, Massachusetts, US)	Mouse IgG1 (1:80; 02-6100; Invitrogen via Thermo Fisher Scientific, Darmstadt, DE)

E-Cadherin	Dako REAL™ Detection System	Mouse IgG1
(1:100; clone NHC-38; DAKO Agilent, Santa Clara, US)	(Biotinylated goat anti-mouse/anti-rabbit immunoglobulins)	(1:1484; 02-6100; Invitrogen <i>via</i> Thermo Fisher Scientific, Darmstadt, DE)
L1CAM	Goat anti-mouse-biotin	Mouse IgG1
(1:1000; clone L1-11A, Peter Altevogt, German Cancer Research Center, Heidelberg, DE)	(1:200; LS-C149505; LS-Bio, Massachusetts, US)	(1:228; 02-6100; Invitrogen <i>via</i> Thermo Fisher Scientific, Darmstadt, DE)
ZEB1	Goat anti-rabbit biotin	Rabbit polyclonal IgG
(1:100; polyclonal; Atlas Antibodies, Bromma, SE)	(1:200; LS-C350860; LS-Bio, Massachusetts, US)	(1:2500; ab37415; Abcam, Cambridge, UK)
ZEB2	Dako REAL™ Detection System	Rabbit polyclonal IgG
(1:100; polyclonal; Atlas Antibodies, Bromma, SE)	(Biotinylated goat anti-mouse/anti-rabbit immunoglobulins)	(1:5000; ab37415; Abcam, Cambridge, UK)
Nestin	Goat anti-mouse biotin	Mouse IgG1
(1:100; clone 10C2; Merck, Darmstadt, DE)	(1:200; LS-C149505; LS-Bio, Massachusetts, US)	(1:100; 02-6100; Invitrogen <i>via</i> Thermo Fisher Scientific, Darmstadt, DE)
SOX2 - brown	Goat anti-rabbit biotin	Rabbit polyclonal IgG
(1:20, polyclonal; Atlas Antibodies, Bromma, SE)	(1:200; LS-C350860; LS-Bio, Massachusetts, US)	(1:2000; ab37415; Abcam, Cambridge, UK)

IHC sections were digitalized (Axioscan Z1, Carl Zeiss AG, Jena, DE) and expressed proteins were analyzed using ZEN3.3 software (Carl Zeiss AG, Jena, DE). To quantify IHC staining of tumors, a tumor score was defined to characterize tumors in terms of percentage of positive stained cells (Frequency) and staining intensity (Intensity). The Frequency score was set from 1-4, Intensity score was set from 1-3 (Table 4).

Table 4: Frequency Score (= % of positively stained cells) and Intensity score (intensity of staining) used for quantification of IHC analysis

Frequency Score	Intensity Score
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1 – Negative/ low (0 – 10%)	1 – Negative/ low
2 – Intermediate (11 – 50%)	2 – Intermediate
3 – High (51 – 90%)	3 – Strong
4 – Strong (> 90%)	–

Statistical analysis

The statistical evaluation was carried out with the help of the program GraphPad Prism 9. Ink (Version 9.5.0 (GraphPad Software by Docmatics, San Diego, California). All data sets were tested for normal distribution using Shapiro-Wilk test.

Parametric data including multiple groups were tested by one-way analysis of variance (one-way ANOVA) for statistical significance. Non-parametrical datasets of multiple groups were analyzed with Kruskal-Wallis one-way ANOVA on ranks test. Statistically significant differences between the groups were assumed at p-values < 0.05 according to Student-Newman-Keuls method (parametric data) and Dunn's method (non-Parametric data), respectively.

Graphs of parametric data were presented by mean with standard deviation or mean with standard error of means (depending on technical and biological replicates), while graphs of non-parametric data were presented by median with (interquartile) range. Students T-test was used to examine two samples of normally distributed data.

Columned data sets were analyzed using one-way-ANOVA plus Tukey's multiple comparisons test for parametric data and Kruskal-Wallis test plus Dunn's multiple comparisons test for non-parametric data. Grouped data sets were analyzed using two-way-ANOVA and Tukey's multiple comparisons test. Growth curve experiments were analyzed *via* nonlinear regression (curve fit) to maintain the growth rate (κ) for logistic growth. Statistical significance was defined with p-value style NEJM at a p-value of < 0.033. Significances are indicated by asterisks: p < 0.033 = *, p < 0.002 = **, p < 0.001 = ***.

Results

Panc1 and Panc89 cell variants exhibit differences in colony formation ability

From the two parental PDAC cell lines (Panc1 and Panc89), representing a heterogeneous cell population with different cell clones, Holo- and Paraclone cell lines were generated via single-cell cloning. First, we comparatively analyzed the colony formation ability, being indicative for the cellular self-renewal properties, of parental Panc1 and Panc89 as well as Holo- and Paraclone cell variants. Focussing on Holo- and Paraclone cells of both PDAC cell lines, the intermediate colony types (Meroclones) were neglected. Typical colony morphologies of Holo-clones and Paraclones in the three cell variants of each PDAC cell line are shown in **Figure 1A**. The total number of colonies formed per well by Panc1 Holo- and Paraclone cells was lower compared to Panc89 Holo- and Paraclone cells, while the total number of colonies formed per well by parental Panc1 and Panc89 cell variants was comparable (12 and 11, respectively). Parental Panc1 cells formed 2 Holo- and 10 Paraclone colonies, while parental Panc89 cells gave rise to 8 Holo- and 3 Paraclone colonies, indicating that in the parental Panc1 cell population Paraclone cells dominate, while in parental Panc89 cells rather Holoclone cells are the dominant cell clone. Panc1 Holoclone cells formed a total of 9 colonies and Panc1 Paraclones formed 11 colonies per well. Contrary, Panc89 Holoclone cells formed a total of 102 colonies, presenting a ~10-fold increase compared to Panc1 Holoclone cells, and Panc89 Paraclones formed 51 colonies per well, still presenting a ~5-fold increase compared to Panc1 Paraclone cells (**Figure 1B**). Regarding the colony type it could be determined that parental Panc1 and Paraclone cells predominately formed Paraclone colonies, while Panc1 Holoclone cells, even though few colonies were formed overall, primarily gave rise to Holoclone colonies (**Figure 1A**). On the other hand, parental Panc89 cells as well as Holo- and Paraclones, primarily formed Holoclone colonies (**Figure 1B**). However, Panc89 Holoclone cells brought up the highest number of colonies ($n = 102$), from which most of the colonies were Holoclones ($n = 96$) and only 6 Paraclone colonies (**Figure 1B**). Compared to these results, Panc1 Holoclone cells formed a total amount of 9 colonies, consisting of about 8 Holoclone colonies and 1 Paraclone colony. Overall, these data indicate a distinct CSC phenotype of both Panc1 and Panc89 Holoclone cells (**Figure 1B**). Furthermore, Panc1 Paraclone cells formed a total of 11 colonies, all of them exhibiting a typical Paraclone morphology, being indicative for their distinct non-CSC phenotype. In contrast, the phenotype of Panc89 Paraclone cells was not that clear as these

cells gave rise to 16 Paraclones but also 35 Holoclones, pointing to a high plasticity of this cell population.

Overall, these findings indicate a high heterogeneity with respect to self-renewal capacity between both parental PDAC cell lines but also between the generated Holo- and Paraclone cells.

Parental Panc1 and Panc89 as well as their derived Holo- and Paraclone cells show distinct transcriptional CSC and EMT signatures

Next, the different cell variants were characterized by transcriptomic analysis. The Principal Component Analysis (PCA) was carried out first **Figure 2A** depicts the PCA for parental Panc1 and Panc89, Holo- and Paraclone cells for the first two principal components, which arranges the samples along the highest (PC1) and orthogonally second highest variation (PC2), respectively. The PCA of the parental Panc1 and Panc89 as well as Holo- and Paraclone cell samples separated all cell variants along PC1 with a variance of about 77%. Holo- and Paraclone populations from both Panc1 and Panc89 separated with a much lower variability of about 7% along PC2. These findings indicate distinct transcriptional signatures for parental Panc1 and Panc89 cells and consistent but smaller gene expression changes between Holo- and Paraclones, irrespective of their origin from Panc1 or Panc89 cells.

Next, a pathway enrichment of sequenced RNA samples from parental Panc1 and Panc89, Holo- and Paraclone cells was performed in order to evaluate differences in the Reactome and stemness pathway activities. Gene sets from the Molecular Signatures Database collection C2 (55), selected for Reactome pathways and a stemness gene set “MUELLER PLURINET” were evaluated *via* Gene Set Enrichment Analysis (GSEA). GSEAs on the differential effect sizes of the Reactome pathways of parental Panc89 cells indicated a stronger Interferon signaling, DNA methylation, and senescence activity compared to parental Panc1 cells. In contrast, parental Panc1 cells showed more cilium assembly, transport, autophagy, and mitotic processes (**Supplementary Figure 1A**). Comparative analysis of Panc1 Holo- and Paraclone samples revealed Holoclone cells to have increased activity of mRNA splicing, DNA repair, chromatin modifying enzymes, transcriptional regulation by TP53, cell cycle checkpoints and Mueller Plurinet, the latter being a strong indicator for stemness properties (56). Panc1 Paraclones, on the other hand, showed increased activity of ECM matrix organization and neutrophil degranulation (**Supplementary Figure 1B**). Panc89 Holoclones were characterized by increased

activity of different mitotic phases, cell cycle checkpoints, organelle biogenesis and maintenance, and transcriptional regulation of TP53 and also Mueller Plurinet, while Panc89 Paraclones were characterized mostly by increased activity for neutrophil degranulation (**Supplementary Figure 1C**). In summary, parental Panc1 and Panc89 cells showed major differences in their pathway activities, but also between the derived Holo- and Paraclone cells distinct differences could be observed.

GSEA performed on the marker genes described for PDAC subtypes according to Moffitt et al. (57) revealed that Panc89 cell variants exhibit gene features related to both the classical-like and basal-like PDAC subtypes, with a stronger tendency towards the basal-like subgroup. Contrary, the expression pattern of Panc1 cell variants could not be clearly assigned to either subtype, but showed a tendency towards the classical subgroup. The transcriptome profile of parental Panc89 cells indicated immune cell pathways from dendritic cells, mast cells or neutrophils. Contrary, the parental Panc1 cells were enriched for cell cycle markers with a significant regulation of mitotic processes (**Supplementary Figure 1D**). Overall, these data indicate that both PDAC cell lines and their derived CSC- and non-CSC population cannot be clearly assigned to the described PDAC subtypes. Next, gene sets that are associated with a positive or negative regulation of the EMT program and invasion properties (58) were comparatively analyzed for all cell variants *via* Gene Set Variation Analysis (GSVA). The analysis revealed a clearly positive regulation of EMT and invasion in parental Panc1 cells, while parental Panc89 cells showed a rather negative regulation of EMT. Panc1 cell lines were further characterized by high invasive properties, reflected by upregulation of genes that positively regulate EMT and Invasion (**Figure 2B**). Further, Panc1 Holoclone cells also showed a trend towards positive EMT regulation, which could not be determined for Panc1 Paraclone cells. Parental Panc89 cells showed an overall low but significant increase of positive regulation of invasion compared to Panc89 Holoclones and Panc89 Paraclones. For the analysis of CSC properties for all analyzed cell variants the Mueller Plurinet gene set was used. Comparing the stemness characteristics in Panc1 cell variants it was found that Panc1 Holoclone cells showed the strongest CSC properties, followed by Panc1 parental cells. Panc1 Paraclone cells showed the weakest CSC characteristics of all Panc1 cell variants. In Panc89 cell variants it could be determined that parental Panc89 cells showed significantly higher CSC activity compared to Panc89 Paraclones, while for CSC activity between parental Panc89 cells and Panc89 Holoclone cells no significant differences could be observed (**Figure 2B**).

In total, 10429 out of 17354 detected genes were found to be differentially regulated between parental Panc1 and Panc89 cells confirming the large sample separation in the PCA (**Figure 2A**). Moreover, clear differences in EMT and CSC characteristics in the analyzed cell variants were determined as exemplified by high expression of *NES*, *VIM*, *L1CAM* and *ZEB1* in Panc1 cells, and *CDH1*, *SOX2* and *OVOL2* in Panc89 cells (**Figure 2C**). In detail, all Panc1 cell variants expressed similar amounts of the mesenchymal markers *VIM* and *L1CAM* and the EMT inducer *ZEB1*. Expression of the CSC-marker *NES* was highest in parental Panc1 cells, followed by Panc1 Holoclones, and lowest in Panc1 Paraclones, with a decreased *NES* expression of about 50% compared to parental Panc1 and Holoclone cells (**Figure 2C**). Contrary, all Panc89 cell variants expressed similar amounts of the epithelial marker *CDH1* (E-cadherin). Furthermore, parental Panc89 and Panc89 Paraclone cells both expressed similar levels of *L1CAM*, while Panc89 Holoclone cells showed only faint *L1CAM* expression. Moreover, Panc89 Paraclones were found to express higher levels of the mesenchymal marker *VIM* and the EMT inducers *ZEB1* and *ZEB2* compared to parental Panc89 and Holoclone cells. Regarding the expression of CSC-markers it was found that parental Panc89 and Panc89 Paraclone cells expressed similar levels of *OVOL2*, whereas *SOX2* was similarly expressed in both parental Panc89 and Panc89 Holoclone cells (**Figure 2C**).

To confirm the transcriptomic differences regarding the EMT and CSC characteristics identified by RNA sequencing, RNA expression of CSC- and EMT-markers was analyzed in parental as well as Holo- and Paraclone cell variants of Panc1 and Panc89 cells by qPCR. Thus, expression of the CSC-markers *NES* (Nestin), *SOX2* and *OVOL2* (ZNF) was analyzed along with the EMT-markers *CDH1* (E-cadherin), *L1CAM*, *VIM* (Vimentin), *ZEB1* and *ZEB2*.

Whereas Nestin expression could not be detected in any cell population of Panc89 cells, it was highly expressed in parental Panc1 and Holoclone cells. In Panc1 Paraclone cells *NES* expression was detectable, but only in low amounts (**Figure 2D**). On the other hand, *SOX2* expression was nearly not detectable in all Panc1 cell lines but *SOX2* was highly expressed in Panc89 Holoclone cells, moderately expressed in parental Panc89 cells and nearly not detectable in Panc89 Paraclone cells (**Figure 2D**). *OVOL2* was higher expressed in all Panc89 cell variants compared to Panc1 cell variants, but with distinct differences between the clones. It was moderately expressed in parental Panc1 and Holoclone cells, but not in Panc1 Paraclone cells. In contrast, *OVOL2* expression was highest in parental Panc89 cells, followed by Paraclone cells and Panc89 Holoclone cells exhibiting the lowest *OVOL2* expression among all Panc89 cell variants

but still higher expression compared to any Panc1 cell line (**Figure 2D**). Next, expression of the epithelial marker *CDH1* was analyzed. While *CDH1* expression was almost undetectable in all Panc1 cell variants, it was clearly expressed in all Panc89 cell variants, not showing any significant differences in the expression level (**Figure 2E**). An inverse expression pattern of mesenchymal markers *L1CAM* and *VIM* was detectable in two PDAC cell lines (**Figure 2E**). All Panc1 cell variants showed higher expression of *L1CAM* and *VIM* compared to Panc89 cell variants, with parental Panc1 cells exhibiting the highest expression of both markers. Compared the Panc89 cell variants, Panc89 Paraclone cells showed the highest expression of either mesenchymal marker compared to parental and Holoclone cells (**Figure 2E**). Finally, expression of the two EMT transcription factors *ZEB1* and *ZEB2* was analyzed. Albeit *ZEB1* expression was generally low in all PDAC cells, it was significantly higher expressed in all Panc1 cell variants compared to Panc89 cell variants (**Figure 2F**). While no differences in the expression level could be determined between the three Panc1 cell variants, Panc89 Paraclone cells exhibited higher *ZEB1* expression compared to parental and Holoclone cells. Regarding *ZEB2* expression, highest expression was detectable in Panc1 Holoclone cells followed by expression in Panc89 Holoclone cells. All other cell variants from Panc1 and Panc89 cells exhibited a rather low expression (**Figure 2F**).

Finally, double IFS were performed to confirm gene expression differences on protein level and to discriminate Holo- and Paraclone cells in the respective parental population. Since Panc1 Holo- and Paraclone cells showed differences in their *NES* and *ZEB2* expression status, while Panc89 Holo- and Paraclone cells clearly differed regarding their expression of *SOX2* and *L1CAM*, these markers were stained in IFS.

The parental Panc1 cell population comprised cells exhibiting both NESTIN and ZEB2 expression but also cells, lacking both markers supporting the view of a heterogeneous cell population consisting of CSC- and non-CSC (**Figure 2G**). In line with the gene expression status, clear differences were determined between Panc1 Holo- and Paraclone cells with Panc1 Holoclone cells being clearly positive for NESTIN and ZEB2, while Panc1 Paraclone cells lacked expression of both markers (**Figure 2G**). Analysis of SOX2 and L1CAM in parental Panc89 cells revealed expression for both markers, but showed no co-expression pattern of SOX2 and L1CAM within the heterogeneous population, as formed colonies were either positive for SOX2 or L1CAM. In contrast, Panc89 Holoclone cells showed SOX2 positive cells which lacked L1CAM

expression, while Panc89 Paraclone cells exhibited strong L1CAM expression but which were SOX2 negative (**Figure 2G**), being well in line with gene expression status.

Overall, these data indicate the existence of different CSC phenotypes which can be associated either with a mesenchymal or an epithelial phenotype. While Panc1 Holoclone cells are characterized by mesenchymal stemness phenotype with pronounced expression of the CSC-marker NESTIN, Panc89 Holoclone cells are characterized by an epithelial stemness phenotype with high SOX2.

Panc89 cell variants show enhanced cell growth rates compared to Panc1 cell populations

Having identified different CSC-EMT-phenotypes, we next intended to analyze the functional behavior of these CSC-phenotypes exhibiting different EMT characteristics in comparison to their parental population and the non-CSC population. Thus, we analyzed the cell growth behavior of parental Panc1 and Panc89 cells as well as their derived Holo- and Paraclone cell lines by measuring cell confluence and nuclei counts over a time period of 168h.

Comparing growth rates of parental Panc1 and Panc89 cells it was found that Panc89 cells grew 43.4% faster than Panc1 cells, with 0.033 k_{Mean} for parental Panc1 and 0.057 k_{Mean} for parental Panc89 cells (**Figure 3A**). Furthermore, also both Panc89 Holo- and Paraclones showed faster cell growth (0.058 k_{Mean} and 0.042 k_{Mean} , respectively) compared to Panc1 Holo- and Paraclone cells (0.031 k_{Mean} and 0.023 k_{Mean} , respectively) (**Figure 3A**). Moreover, Holoclone cells of either cell line grew faster compared to the respective Paraclone cells. Growth of Panc1 Holoclones was increased by 25.6% compared to Panc1 Paraclone cells, Panc89 Holoclone cells grew 27.7% faster than Panc89 Paraclones, pointing to a fast proliferative character of the epithelial CSC. Finally, parental Panc1 and Panc89 cell populations grew faster than their derived Holo- and Paraclone cells, despite parental Panc89 cells which showed approximately the same growth rate than Panc89 Holoclone cells (**Figure 3A**). Overall, these data underscore that mesenchymal PDAC cells exhibit a slowly growing phenotype compared to epithelial PDAC cells which also applies to the CSC-fraction.

Panc1 Holoclone cells are less migratory but highly invasive in a mesenchymal invasion manner, while Panc89 Holoclone cells show pronounced cell migration but slow invasion in clusters

Next, we analyzed the migratory behavior of parental Panc1 and Panc89 and their derived Holo- and Paraclone cells. After 8 h, gap cell confluence was less than 20% for all Panc1 cell variants with no differences between parental, Holo- and Paraclone cells (**Figure 3B**). In contrast, gap cell confluences of all Panc89 cell variants were much higher with Panc89 Holoclone cells leading to the highest cell confluence on gap of 75%. Panc89 Paraclone cells showed only 25% cell confluence while parental Panc89 cells showed about 35% cell confluence on gap, indicating that epithelial Panc89 cells migrated faster than mesenchymal Panc1 cells and particularly the epithelial CSC fraction exhibited the highest migration potential (**Figure 3B**). Besides the clear differences in the migration velocity, Panc1 and Panc89 cell lines also used different modes of migration fitting together with the different EMT phenotypes. While Panc89 Holoclone cells migrated in cell clusters with high cell-cell contact, Panc1 Holoclone cells exhibited a mesenchymal-like migration of single cells (**Figure 3C**).

To analyze invasive properties, all PDAC cell lines were seeded in ULA plates to form spheroids. Initial tests revealed that cell invasion of Panc1 cells is much faster than those of Panc89 cell variants, resulting in an invasion monitoring time of 3 days for Panc1 cell variants and 7 days for Panc89 cell variants. These findings are well in line with the results from GSEA analysis on invasion associated genes, indicating high invasive activity Panc1 cells compared to Panc89 cells (**Figure 1I**). Accordingly, Panc1 cell populations formed more aggregate like spheroids indicating lower amounts of tight cell-cell contacts, while Panc89 cell populations formed compact spheroids leading to smaller spheroids of all Panc89 cell variants compared to Panc1 cell populations (**Figure 3D**). Moreover, the mode of cell invasion clearly differed between Panc1 and Panc89 cells. While Panc1 cell variants showed single cell invasion or invasion in chains of single cells, all Panc89 cell variants invaded as clusters comparable to tumor cell buds (59). Spheroid sizes of all Panc1 cell variants were about 1000 μm at day 0 and did not considerably change until day 3. In contrast, all Panc89 cell variants formed spheroids of about 300 μm on day 0 which further increased in size up to 500 μm at day 7 (**Figure 3D+E**). Moreover, huge differences in formation of invasive fronts were detected. While Panc1 cell populations already showed 20 invasive fronts at day 1, with no clear differences between parental, Holo-

and Paraclone cells, Panc89 cell populations only exhibited 5 invasive fronts at day 5, which not further changed until day 7 (**Figure 3F**). Interestingly, the number of invasion fronts declined in Panc1 cells, being mostly pronounced in the Panc1 Holoclone cells. However, invasion distances concomitantly increased in Panc1 cells, rising from 260-350 μm on day 1 to about 400-500 μm at day 3. In line with the results of the invasion fronts, the most pronounced increase of the invasion distance from day 0 to day 3 was observed in Panc1 Holoclone cells. In contrast, invasion distances of all Panc89 cell variants were much shorter (about 100 μm) and did not change over time (**Figure 3G**).

Despite the significantly higher cell migration of Panc89 Holoclone cells compared to Panc89 Paraclone cells, no clear differences regarding cell invasion were observed between the respective Holo- and Paraclone cell populations, also applying to the Panc1 cell populations. Overall, these data underscore that the EMT and not the CSC phenotype predominantly determines the migration and invasion mode of PDAC cells. Thus, these data underscore that mesenchymal Panc1 cells use a mesenchymal mode of single cell migration and invasion, while Panc89 cells exhibiting an epithelial phenotype use rather a cluster-like migration and invasion mode.

Panc1 and Panc89 cell variants only marginally differ regarding organ specific adhesion

As PDAC metastasizes predominantly in liver, lung and peritoneum (4–6), it was next analyzed whether the different PDAC CSC populations differ with respect to their adhesion properties to different organs. After 4h, about 70% Panc1 Paraclone cells adhered to the liver endothelial TMNK-1 cells, which was significantly more than in Panc1 parental and Holoclone cells. Cell adhesion of all Panc89 cell variants was comparable to those of Panc1 parental and Holoclone cells of about 60% (**Figure 3H**). The largest difference in adhesion behavior could be detected for adhesion to lung endothelium. While 60% of Panc1 parental, Holo- or Paraclone cells adhered to HuLEC-5a cells, cell adhesion of about 80% of all Panc89 cell variants could be observed. Regarding cell adhesion to mesothelial Met-5a cells no differences could be detected for all PDAC cell lines showing 75-80% adhered cells after 4 h (**Figure 3H**). Overall, these findings indicate that the EMT- and CSC- phenotype of PDAC cells rather marginally impact cell adhesion to organ specific endothelial cells, despite that Panc1 Paraclone cells showed a

better adhesion to liver endothelial cells and Panc89 cells with a partial EMT cells showed a better adhesion to lung endothelial cells compared to mesenchymal Panc1 cells.

Panc1 and Panc89 cell clones essentially differ with respect to their metastatic capacity *in vivo*

Finally, to assess whether the CSC-/EMT-phenotypes and the associated functional differences identified *in vitro*, are related to a difference in their metastatic behavior, a tumorigenicity assay with intrasplenic inoculation of either Panc1 and Panc89 Holo- or Paraclone cells was performed.

As shown in **Figure 4A**, animals inoculated with Panc89 Holo- or Paraclone cells survived much shorter than animals inoculated with Panc1 Holo- or Paraclone cells. The median survival of mice inoculated with Panc89 Holoclone cells was 74 days and 66 days for Panc89 Paraclone cells. In comparison, animals inoculated either with Panc1 Holo- or Paraclone cells showed about twice as long median survival of 146 days (**Figure 4A**). Of note, animals inoculated with Panc1 cell variants were not killed because of the reduced health status due to the tumor burden, rather than that tumor progression was very slow so that the experiment was terminated. Moreover, no significant differences in survival could be determined between Holo- and Paraclone cells of the distinct PDAC cell lines.

Examination of the macroscopic tumor manifestation revealed clear differences between Panc1 and Panc89 cell variants as well as between Holo- and Paraclone cells. Ultrasound examination revealed macroscopic tumor formation in 7/10 animals with a median tumor area of 11.68 mm³ for Panc1 Holoclone tumors and in 2/10 animals for Panc1 Paraclones (with no median macroscopic tumor area detectable because one of two tumors could not be measured *via* ultrasound). In contrast, in 10/10 animals inoculated with Panc89 Holoclone cells, tumors could be detected with a median tumor area of 137.9 mm³ and in 7/10 animals with a median macroscopic tumor area of 122.1 mm³ for Panc89 Paraclone tumors (**Figure 4B, C**). Thus, the shorter survival times of animals inoculated with Panc89 cell variants was associated with higher tumor burden compared to Panc1 Holo- and Paraclone cells.

Moreover, Panc1 Holo- and Paraclone tumors were predominantly found in the pancreas, but also in liver and lung, while Panc89 Holo- and Paraclone tumors were predominantly detectable in the peritoneum, followed by pancreas, liver and lung (**Figure 4C**). In detail, intrasplenic

inoculation of Panc1 Holoclone cells led to clearly more tumors compared to Panc1 Paraclone tumors, which were predominantly formed in pancreas (5/10 animals) and liver (3/10 animals), while inoculation of Panc1 Paraclone cells only led to tumors in pancreas and lung of 1/10 animals (**Figure 4C**). Macroscopically, inoculation of Panc89 Holoclones also led to higher number of tumors compared to Panc89 Paraclone cells and also compared to all Panc1 cell variants. Panc89 Holoclone tumors were found in the peritoneum of 8/10 animals, in the pancreas of 2/10 animals and in the liver and lung of 1/10 animals. Panc89 Paraclone tumors were found in the peritoneum of 4/10 animals, in the pancreas of 2/10 animals and in the liver of 1/10 animal (**Figure 4C**).

Having determined clear differences regarding the frequency and site of metastases of the differentially inoculated animals, pancreatic, liver, lung and peritoneal tissues were stained for human PanCK (**Supplementary Figure 2A**) to assess the microscopical metastatic burden in terms of size and number of formed microscopic tumors.

The size of the microscopic tumors was determined by measuring the PanCK positive areas in the tissues. In line with the size of the macroscopic tumors, the median tumor areas of both Panc89 Holo- and Paraclone tumors were larger than Panc1 Holo- and Paraclone tumors (**Figure 4D**). Inoculation of Panc89 Paraclone cells led to the largest microscopic tumors area with a median tumor area of about 25 mm², followed by Panc89 Holoclone tumors with a median tumor area of about 8 mm². In contrast, Panc1 Holo- and Paraclone cells formed tumors with a median tumor area of less than 5 mm² (**Figure 4D**). Besides it could be noted that 5/8 Panc89 Paraclone tumors were characterized by a pronounced cyst formation (supplementary **Figure 2B**).

Furthermore, intrasplenic inoculation of Panc1 Holoclone cells led to a total of 31 microscopic lesions in 9/10 animals, while only 9 microscopic tumors were found in 8/10 animals inoculated with Panc1 Paraclone cells. Of note, the highest number of microscopic Panc1 Holoclone tumors were found in the pancreas (22/31), while Panc1 Paraclone lesions were mostly found at other sites (brown fat tissue, duodenum, undefinable in 5/9 metastatic lesions) (**Figure E**). With 17 microscopic tumors in 10/10 mice the number of microscopic Panc89 Holoclone tumors was much lower compared to Panc1 Holoclones, and organ manifestation was also very different as most tumors were found in the peritoneum (14/17 metastatic lesions) like the macroscopic tumors (**Figure E**). The number of animals with Panc89 Paraclone tumors and the total number of microscopic lesions were similar to Panc1 Paraclone cells. Inoculation of

Panc89 Paraclone cells gave rise to 8 microscopic tumors in 7/10 animals (**Figure E**). Here, a slightly higher prevalence of tumor formation in the peritoneum compared to other sites was detectable (3/8 lesions).

Overall, inoculation of Panc89 Holo- and Paraclone cells led to a more pronounced metastatic burden in terms of larger metastases which predominantly manifested in the peritoneum compared to Panc1 Holo- and Paraclone cells which led to smaller tumors mostly in the pancreas. Higher tumor burden after inoculation of Panc89 Holo- and Paraclone cells was associated with significantly shorter survival times. However, inoculation of Panc1 Holoclone cells led to the highest total number of tumoral lesions being in line with the more pronounced invasive phenotype of the cells. The fact that Panc89 Paraclone cells also led to a pronounced tumor burden which was not observed for the two Panc1 cell variants underscores a higher plasticity of these cells. Altogether, these findings indicate clear differences in the metastatic capacity between mesenchymal and epithelial/hybrid CSC populations.

Panc1 and Panc89 Holo- and Paraclone tumors exhibit differences in EMT- and CSC- marker expression

To verify the *in vitro* identified CSC-/EMT phenotypes of Panc1 and Panc89 Holo- and Paraclone cells *in vivo*, IHC staining of resected tissues was performed to analyze the expression of the CSC-markers NESTIN and SOX2 as well as the EMT-markers E-cadherin, L1CAM, ZEB1 and ZEB2. To identify human PDAC cells, staining of human PanCK was performed. PanCK staining of tumors revealed different morphologies of Panc1 and Panc89 Holo- and Paraclone tumors. While Panc1 Holo- and Paraclone tumors were characterized by scattered arrangement of single tumor cells, Panc89 Holo and Paraclone tumors showed the arrangement of positively stained cell clusters. Especially Panc89 Holoclone tumors presented distinct PanCK positive cell clusters, surrounded by tumor stroma. Cell clusters in Panc89 Paraclone tumors were larger, with a less intense PanCK staining, but also infiltrated with huge tumor stromal areas (**Supplementary Figure 2A**).

Confirming gene expression data, Panc1 Holoclone tumors showed negative to low expression of E-cadherin in analyzed tissues with a Frequency and Intensity score of around 1 (**Figure 5A**). Panc1 Paraclone tumors showed a slight but significant increase of E-cadherin expression with a Frequency- and Intensity score of 2 compared to Panc1 Holoclone tumor tissues (**Figure 5A**).

In Panc89 Holo- and Paraclone tumors E-cadherin was highly expressed (Frequency and Intensity score of about 4) with no considerable differences between Holoclone and Paraclone tumors (**Figure 5A**).

L1CAM expression of Panc1 Holoclone tumors was scored with a Frequency of around 2 (Intensity score 1), while Panc1 Paraclone cells showed a significantly lower L1CAM expression (Frequency- and Intensity score 1, **Figure 5B**). In contrast, Panc89 Holoclone tumors showed a very faint expression of L1CAM (both Frequency and Intensity score of 1) compared to Panc89 Paraclone tumors, exhibiting the highest L1CAM expression of all tumors analyzed (Frequency and Intensity score of 3 and 2) (**Figure 5B**). However, in some Panc89 Holoclone tumors, L1CAM expression could be noted (**Supplementary Figure 3**).

IHC analysis of ZEB1 in Panc1 and Panc89 Holo- and Paraclone tumors revealed that Panc1 Holoclone tumors exhibited strong ZEB1 expression (Frequency and Intensity score 4) while Panc1 Paraclone tumors were characterized by low to negative ZEB1 expression (**Figure 5C**), which was not in line with the *ZEB1* gene expression of the cells *in vitro* demonstrating similar RNA levels of *ZEB1* in both Panc1 cell variants. In both Panc89 Holo- and Paraclone tumors high ZEB1 expression with a Frequency and Intensity score of 3-4 was noted (**Figure 5C**), which also differed from absent *ZEB1* gene expression in Panc89 Holo- and Paraclone cells *in vitro*. Of note, ZEB1 and ZEB2 expression were mostly located in the nuclei indicative for their functional activity, and both markers could not only be detected in PDAC cells but also in stroma cells. Panc1 Holoclone tumors showed a significantly higher expression of ZEB2 (Frequency score of 2-3 and Intensity score of 1) compared to Panc1 Paraclone tumors exhibiting no or only weak expression of ZEB2 (Frequency and Intensity score of 1, **Figure 5D**). Panc89 Holoclone tumors showed a slightly lower ZEB2 expression (Frequency and Intensity score 3/2) compared to Panc89 Paraclone tumors with a Frequency and Intensity score of 4/3 (**Figure 5D**).

Finally, expression of the CSC-marker NESTIN and SOX2 was assessed in all tumors. In Panc1 Holoclone tumors NESTIN was strongly expressed (Frequency score 2-3 and Intensity score 4), significantly differing from Panc1 Paraclone tumors which showed no or only weak NESTIN expression (Frequency- and Intensity score 1), which well reflected the different expression pattern identified *in vitro*. In line with our *in vitro* findings both Panc89 Holoclone and Paraclone tumors showed no or only very faint NESTIN expression (**Figure 5E**).

In contrast to the *in vitro* findings, SOX2 expression could be detected in Panc1 Holoclone tumors (Frequency and Intensity score of 2/3) and expression levels were higher compared to Panc1 Paraclone tumors (Frequency and Intensity Score of 1/2, **Figure 5F**). The expression of SOX2 in Panc89 Holoclone tumors was also significantly higher (Frequency and Intensity Score of 2/2) compared to Panc89 Paraclone tumors (Frequency and Intensity Score of 1/1) (**Figure 5F**).

As mentioned above, 4/17 Pan89 Holoclone tumors showed areas of L1CAM expression. When correlating L1CAM and SOX2 expression in this group, no significant correlation could be noted. However, analyzing Panc89 Holoclone tumors expressing L1CAM (Frequency score of 2-3) regarding SOX2, L1CAM expression in these tumors was significantly associated with low/absent SOX2 expression (Frequency score of 1) (**Supplementary Figure 3**) being indicative of the phenotype of Panc89 Paraclone cells. By examining the tissues of origin of these tumors it could be found that one tumor originated directly from the pancreas. The other examined tumors could not be clearly assigned to one organ but were all surrounded by fat tissue, indicating localization of the tumors in the peritoneum.

In summary, IHC analysis widely confirmed the gene expression profile of the cells identified *in vitro*, approving mesenchymal Panc1 Holoclone cells with a Nestin-dominated CSC phenotype, while mesenchymal Panc1 Paraclone cells showing no stemness characteristics. Contrary, Panc89 Holoclone cells exhibited mostly an epithelial SOX2-dominated CSC phenotype, whereas Panc89 Paraclone cells exhibited a partial/hybrid EMT cell phenotype with less CSC marker expression. However, certain variations from the expression patterns identified *in vitro* could be noted in Panc89 Holo- and Paraclone tumors (e.g., L1CAM, SOX2, Zeb1 and Zeb2) but also in Panc1 Holoclone cells (Sox2) underscoring the plasticity of the cells which is influenced by the tumor microenvironment.

Overall, these data indicate that the different CSC-/EMT-phenotypes of PDAC cell populations might yield different metastatic manifestations being associated with different survival times.

Discussion

Cancer cell plasticity and tumor heterogeneity, e.g. manifested by the presence and interplay of CSCs and non-CSC populations, along with the pronounced tumor stroma are associated with poor prognosis and increased resistance to chemotherapy of PDAC (60–72). Besides, CSCs and EMT are both linked to cancer progression and early metastasis, also in PDAC (18–20,73). CSCs are characterized by the unique ability to self-renewal, initiation of tumoral lesions at primary and secondary sites, the capability to resist cell death induction and to give rise to plastic, highly proliferative transit amplifying progenitor cell populations (21–23,74–78). As they also generate more or less differentiated cancer cells, CSCs are one important origin of tumor cell heterogeneity (20,24–27,79). The link between EMT and MET processes and CSCs has been already shown (18–20,73), however, it is still poorly understood whether CSC and EMT phenotypes are always interconnected, how CSC phenotypes of epithelial and mesenchymal tumor cells are characterized and whether these might be related to different functional malignant outcomes. To gain a better understanding of plasticity in PDAC with a particular focus on CSCs and EMT and its contribution to malignancy associated properties, two different PDAC cell models were comparatively analyzed. Mesenchymal Panc1 cells derived from a primary PDAC have presumably already undergone EMT, while Panc89 cells originate from a lymph node metastasis and having most likely undergone EMT and MET leading to metastatic outgrowth in the lymph node (80). Isolated Panc1 and Panc89 Holo- and Paraclone cells, as well as the two related parental cell populations were comprehensively analyzed by expression and functional analysis *in vitro* and *in vivo*. First, CFAs and expression analysis of CSC markers revealed different CSC phenotypes in the two PDAC cell lines. While Panc1 Holo-clone cells were characterized by high Nestin expression and absent SOX2 expression, Panc89 Holoclone cells exhibited an inverse expression pattern showing a SOX2 dominated phenotype. Moreover, the CSC phenotype of Panc89 Holoclones was associated with a higher colony formation ability compared to Panc1 Holoclones, indicating a more pronounced self-renewal capacity of these cells. Of note, pathways involved in the activity of cell cycle checkpoints, transcriptional regulation of TP53 and Mueller Plurinet seemed to play a role in the PDAC CSC populations, as these genes were upregulated in both Panc1 and Panc89 Holoclone cells compared to related Paraclones. Mueller Plurinet is a protein-protein network shared by pluripotent cells and has become a prominent classifying system for pluripotency and self-renewal properties (56). Activation of TP53 and cell cycle checkpoints are interconnected since TP53

enables dividing cells to repair anomalies before proceeding through cell cycle checkpoints, acting as a tumor suppressor (81–84). Therefore, mutations in TP53 are strongly associated with tumor progression in various human cancers, also PDAC, and recent reports have shown its impact on stemness properties in cancer cells (80–91). Overall, these data support the existence of distinct CSC phenotypes of Panc1 and Panc89 Holoclones.

Furthermore, clear differences with respect to the EMT phenotype were noted. Parental Panc89 and Panc89 Holoclone cells were predominantly characterized by an epithelial phenotype exemplified by high E-cadherin expression and concomitant low expression of mesenchymal markers (Vimentin, L1CAM, ZEB1 and ZEB2). In contrast, all Panc1 cell variants were characterized by low E-cadherin expression and marked expression of mesenchymal markers underscoring the mesenchymal phenotype. As Panc89 Paraclone cells showed high E-cadherin expression, low Vimentin expression but also considerable expression of L1CAM, ZEB1 and ZEB2 which was higher compared to the other Panc89 cell variants but still lower compared to Panc1 cell variants (despite ZEB2), these cells seemed to exhibit a partial/hybrid EMT phenotype (92–95). These phenotypes were widely confirmed by IHC staining of the tumors formed *in vivo* by the inoculated Holo- and Paraclone cell populations. However, in Panc89 Holoclone tumors areas with high L1CAM expression and low SOX2 expression but also with ZEB1 or ZEB2 expression were noted, and Panc1 Holoclone tumors showed elevated SOX2 expression which was absent in Panc1 Holoclone cells *in vitro*. This might be due to the fact that SOX2 functions as a stability factor in the control of PDAC cell proliferation, being required for growth of PDAC cells. Apparently SOX2 needs to be stably expressed, as increased as well as decreased levels of SOX2 reduces tumor growth of PDAC *in vivo* and *in vivo* (96). These findings support our data showing that a gain in SOX2 expression in Panc89 and Panc1 Holoclone tumors might be crucial to ensure tumor outgrowth. Altogether, these data indicate a phenotypic switch of the tumor cells presumably forced by the environmental factors the tumor cells are exposed to *in vivo*. Hence, myofibroblasts and macrophages, both important stroma cell populations in pancreatic tumors and metastases (97,98), were already shown to induce L1CAM expression in PDAC cells (99,100).

Even though the genomic profiling revealed both Panc1 and Panc89 Holoclone cells to show similarities in TP53 and cell cycle checkpoint regulation as well as stemness associated Mueller Plurinet activity, we could identify two distinct CSC phenotypes: a mesenchymal Nestin-dominated (Panc1 Holoclone cells) and an epithelial SOX2-dominated CSC phenotype (Panc89

Holoclone cells). Of note, both phenotypes were associated with clearly different functional properties. Besides the more pronounced colony formation, Panc89 Holoclone cells showed a clearly faster growth behavior compared to Panc89 Paraclone cells but also to Panc1 Holoclone cells, being in line with the fact that proliferative activity of epithelial cells is generally higher than those of mesenchymal cells (101,102). In contrast, the latter cells exhibited a faster cell invasion in a typical mesenchymal cell manner with increased numbers of invasive fronts and longer invasion distances, underscored by the activity of EMT and invasion associated genes shown by the transcriptomic profile. In contrast, Panc89 Holoclone cells used a cluster mode of slow cell invasion, again fitting together with the epithelial phenotype of these cells (80,101,102). Also in line with these findings, expression of *VIM* (103–108) and *L1CAM* (99,109–111) are associated with an increased invasive potential in a variety of cancers, also in PDAC cells.

Moreover, these distinct growth and invasive properties were linked to different metastasis *in vivo*. Accordingly, Panc1 Holoclone cells exhibiting the strongest invasive potential using a mesenchymal invasion mode led to the highest number of metastatic lesions predominantly in the pancreas compared to epithelial Panc89 cell variants and Panc1 Paraclones. The fact that Panc1 Holoclone cells led to formation of more and larger tumors compared to Panc1 Paraclones underscores that the presence of CSC properties in mesenchymal PDAC cells is prerequisite for metastatic tumor formation.

However, inoculation of Panc89 Holoclones led the most pronounced macroscopic tumor burden with the highest number and the largest tumors compared to all other cell variants, albeit the differences between Panc89 Holo- and Paraclones were not that pronounced, also with respect to survival times, pointing again to a microenvironment mediated phenotypic switch of the tumor cells (see above). Of note, tumor formation of Panc1 and Panc89 Paraclone cells suggested that non-CSC variants can gain CSC properties, since both Paraclone variants were able to induce tumor formation, although not as pronounced as Panc1 and Panc89 Holoclone cells. Despite this gain of CSC properties in Panc1 and Panc89 Paraclone cells, expression of CSC markers *in vivo* remained low in these tumors.

In contrast to Panc1 tumors, tumors formed by Panc89 cell variants predominantly manifested in the peritoneum. Adhesion assays to organ specific endothelial cells did not reveal any considerable differences between Holo- and Paraclone populations of either cell line but indicated that Panc89 cell variants better adhere to lung endothelium than Panc1 cell variants. However,

these results were not in line with tumor manifestation patterns *in vivo* which might indicate that the different organ colonization and metastatic outgrowth of the inoculated cell populations more rely on other cell properties as well as the local microenvironment in the organ than adhesion to the organ specific endothelium. Moreover, CSC properties do not seem to determine homing to secondary sites rather than dissemination mode and outgrowth of the tumor cells.

As outlined before, both Nestin and SOX2 have been associated with CSC phenotypes and were linked to malignancy associated properties. Thus, in a murine PDAC model, shRNA mediated reduction of *NES* expression led to decreased tumor volume and hepatic metastases (37,38), being in line with our results demonstrating that Nestin expressing Panc1 Holoclone cells showed higher self-renewal capacity and invasive properties *in vitro* and formed higher number of tumors *in vivo* compared to Panc1 Paraclone cells. In contrast, Panc89 Holoclones lacked Nestin expression, but were characterized by high SOX2 expression compared to Panc89 Paraclone cells, as well as compared to all Panc1 cell variants. However, *in vivo* Panc1 and Panc89 Holoclone tumors both exhibited similar amounts of SOX2, supporting the role of the microenvironment as determining factor of CSC properties (20,24–27,112). Of note, elevated SOX2 expression in Panc89 Holoclones was associated with the formation of the highest number of colonies and the fastest growth rate *in vitro* and the formation of the largest tumors *in vivo*. Furthermore, it can be speculated that elevation of SOX2 might also be involved in outgrowth of Panc1 Holoclone tumors (32,96,113).

PDAC cells are known to predominantly metastasize to the liver, lung and the peritoneum (4–6). As described above, the highest number of Panc1 Holoclone tumors was predominantly found in the pancreas, while Panc89 Holoclone cells formed most tumors in the peritoneum. The fact that mesenchymal Panc1 cells are derived from the primary tumor, together with their gene activity associated with EMT and invasion, suggests that these cells have undergone EMT to leave the primary tumor. However, whether these cells would ever have been able to form metastases in this patient remains unsolved. Furthermore, it may explain, why most Panc1 Holoclone tumors were found in the pancreas indicating that these cells are still optimally adapted to their original tissue. In contrast, epithelial Panc89 cells originate from a lymph node metastasis, thus these cells have proven their disseminating potential to leave the primary tumor (either after EMT using a mesenchymal invasion mode or while maintaining

an epithelial/hybrid cell stage using a cluster-like mode) and to grow out as metastasis at a secondary site (e.g., after MET).

Conclusions

In summary, these results support the view that mesenchymal CSC have a higher propensity to spread and the ability to colonize secondary sites (exemplified by the highest number of total tumoral lesions), which however does not ultimately lead to immediate disease progression and short survival. In contrast, an epithelial CSC phenotype seemed to be associated with slow cell invasion but the concomitant advantage for rapid tumor outgrowth, resulting in a rapid increase of a life-threatening tumor burden (exemplified by the highest number of macroscopic tumors and shorter survival). Overall, our data support the view that different CSC phenotypes exist in PDAC that are associated with distinct EMT/MET phenotypes of PDAC cells essentially determining PDAC cell fate and function.

Abbreviations

CAF: Cancer associated fibroblasts; **CFA:** Colony formation assay; **CRC:** Colorectal cancer; **CSC:** Cancer stem cell; **DRA:** Dose-Response-Analysis; **EMT:** Epithelial-Mesenchymal-Transition; **FCS:** Fetal calf serum; **GAPDH:** Glycerinaldehyde-3-phosphate-Dehydrogenase; **Holo:** Holo-clone cells; **IF:** Intermediate filament; **IFS:** Immunofluorescent staining; **IHC:** Immunohistochemical staining; **k:** growth rate; **KMS:** Kaplan-Meier-Survival analysis; **MET:** Mesenchymal-Epithelial-Transition; **Para:** Paraclone cells; **PCR:** Polymerase chain-reaction; **PDAC:** Pancreatic ductal adenocarcinoma; **qPCR:** Quantitative polymerase chain-reaction; **SC:** Stem cell; **SCC:** Single-cell cloning; **TF:** Transcription factor

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Data availability:

1. The datasets generated and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS]
2. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.
3. All data generated or analysed during this study are included in this published article [and its supplementary information files].

Conflicts of interest

The authors declare no conflicts of interest.

Author's contributions

Conceptualization: LMP and SSe; Investigation: LMP, U-UY, AS, A?, A-SM, CH, J-PG, OW, SF, HK, US, HB; Resources: US, SF, HB, SSe; Writing—original draft preparation: LMP, SSe; Writing—review and editing: all authors; Visualization: LMP; Funding acquisition: SF, SSe. All authors contributed to the article and approved the submitted version.

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Figure legends

Figure 1 Panc1 and Panc89 cell variants exhibit differences in their colony formation ability.

Parental Panc1 and Panc89 as well as Holo- and Paraclone cells were analyzed regarding their colony formation ability *via* CFA. 400 cells of each cell line were seeded in duplicates/triplicates in 6-well plates and after eight to eleven days, the colonies were fixed with paraformaldehyde and stained with crystal violet. The colonies formed were morphologically characterized regarding Holo- and Paraclones. **A)** Representative images of colonies formed by parental Panc1 and Panc89 as well as their derived Holo- and Paraclone cells. Scale bar = 100 μ m **B)** Number of formed Holo- and Paraclones per well formed by parental Panc1, Holo- and Paraclone cells (left) as well as parental Panc89, Holo- and Paraclone cells (right). Data are shown as mean with SEM from 3 independent experiments 3. $p < 0.033 = *$

Figure 2 Panc1 and Panc89 cell variants show distinct transcriptional CSC and EMT signatures.

A) Principal Component Analysis (PCA) based on the 10% most varying transcripts across all of parental Panc1 and Panc89 cells as well as their derived Holo- and Paraclone cells using the R package PCAtools (v2.10.0). PCA was performed for the highest variation in the first principal component (PC1) on the X-axis, and for the lower variations by the second principal component (PC2) on the Y-axis. All cell variants are denoted by color (red = Panc1 cell variants, blue = Panc89 cell variants) and symbol, respectively. **B)** Gene Set Variation Analysis (GSVA) of gene sets associated with positive or negative regulation of the EMT program, invasion properties and stemness (by Mueller Plurinet gene set) in parental Panc1 and Panc89 as well as their Holo- and Paraclone cells. The numbering of all cell variants represents the number of the independent replicate. **C)** Transcriptomic analysis revealed differences in the expression of the indicated CSC- (*NES*, *OVOL2*, and *SOX2*) and EMT- (*CDH1*, *VIM*, *L1CAM*, *ZEB1* and *ZEB2*) in parental Panc1 and Panc89 as well as Holo- and Paraclone cells. Data are represented as means with SD. Gene expression analysis by qPCR was performed for the CSC-marker **D)** *NES*, *SOX2* and *OVOL2*, for the EMT-marker **E)** *CDH1*, *L1CAM* and *VIM* as well as for EMT-inducers **F)** *ZEB1* and *ZEB2*. Data are represented as mean with SD or median with interquartile range. $p < 0.033 = *$, $p < 0.002 = **$, $p < 0.001 = ***$. **G)** Representative images of immunofluorescent staining of NESTIN and ZEB2 in Panc1 cell variants and of SOX2 and L1CAM

in Panc89 cell lines. Cell nuclei were stained with Hoechst. Scale bar = 100 μm . Data are shown from 3 independent experiments.

Figure 3 Panc1 and Panc89 cell variants show different characteristics regarding cell growth, migration and invasion. A) Cell growth rate analysis of parental Panc1 and Panc89, as well as Holo- and Paraclone cells over 168h. Data are depicted as percent [%] cell confluence and total number [#] of nuclei. Analysis of k for cell confluence and nuclei count was performed by logistic growth analysis. **B)** Migration ability of Panc1 and Panc89 cell variants was determined by monitoring the cell confluence on a gap after 8h. **C)** Representative images of cell migration of the different cell variants after 8h. Typical cluster like migration of parental Panc89 and Holoclone cells is marked. Scale bar= 200 μm . **D)** Representative images of spheroid formation and invasion ability at day 0 and day 3 (for Panc1 cell variants) and day 7 (for Panc89 cell variants). Typical invasion modes of mesenchymal Panc1 cell variants and epithelial Panc89 cell variants are marked. Scale bar= 200 μm **E)** Spheroid size [μm], **F)** number of invasive fronts and **F)** the invasion distance [μm] of Panc1 cell variants (day 1, day 3) and Panc89 cell variants (day 5, day 7). **H)** Percentage of adhered Panc1 and Panc89 cell variants to liver endothelial cells (TMNK1), lung endothelial cells (HuLEC-5a) and mesothelial cells (Met-5a) after 4h of cell adhesion. Graphs represent mean with SEM or median with interquartile range. $p < 0.033 = *$, $p < 0.002 = **$, $p < 0.001 = ***$

Figure 4 Panc1 and Panc89 cell clones differ with respect to their metastatic capacity *in vivo*. SCID beige mice were inoculated intrasplenically with either 1×10^4 Panc1 Holo- or Paraclone cells or Panc89 Holo- or Paraclone cells (10 mice/group). **A)** Median survival at post-operative day (POD) after tumor cell inoculation by Kaplan-Meier-Survival analysis. p-value of < 0.001 (***). **B)** Tumor area [mm^3] of macroscopic tumors and **C)** number of animals with macroscopic tumors formed in pancreas, liver, lung and peritoneum. **D)** Tumor area [mm^2] of microscopic tumors and **E)** number of animals with microscopic tumors in the examined organs. **B+D)** Data are presented as median with interquartile range. Holo = Holoclone cells, Para = Paraclone cells

Figure 5 Panc1 and Panc89 Holo- and Paraclone tumors exhibit differences in EMT and CSC marker expression. SCID beige mice were inoculated intrasplenically with either 1×10^4 Panc1 Holo- or Paraclone cells or Panc89 Holo- or Paraclone cells (10 mice/group). Resected tissues and tumor lesions were stained for **A)** E-cadherin, **B)** L1CAM, **C)** ZEB1, **D)** ZEB2, **E)** Nestin and **F)** SOX2. Representative pictures of stained tissues of Panc1 and Panc89 Holo- or Paraclone tumors are presented. Scale bar = 100 μ m. Right panels show the statistical analysis of the respective frequency and intensity scores of positively stained cells. Data are represented as median with range in violin plots $p < 0.033 = *$, $p < 0.002 = **$, $p < 0.001 = ***$

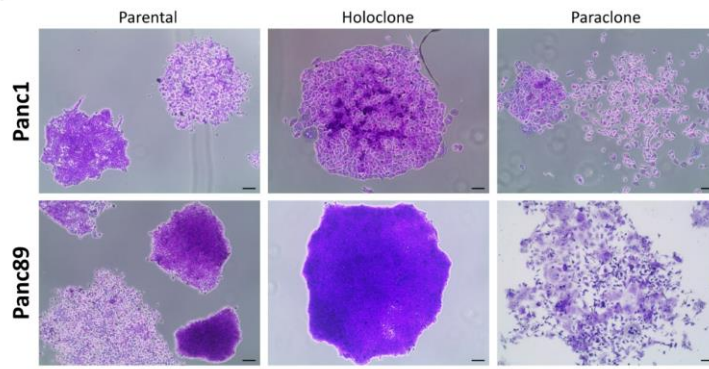
Supplementary Figures

Supplementary Figure 1 Transcriptomic analysis of Panc1 and Panc89 cell variants revealed differences in their pathways activities. Comparative Gene Set Enrichment Analysis (GSEA) on the differential effect sizes of the reactome pathways in **A)** parental Panc1 and Panc89 cells, **B)** Panc1 Holo- and Paraclone cells and **C)** Panc89 Holo- and Paraclone cells. GSEA on the shrunken log2 fold changes from apeglm (53) was performed using GAGE (v2.48.0) (54) against Hallmark, Reactome and Gene Ontology Biological Processes (GOBP) gene sets extracted from the msigdb R package (v7.5.1). **D)** RNA sequencing-based subtyping of parental Panc1 and Panc89, Holo- and Paraclone cells. according to the classical-like and basal-like PDAC cell subtypes of Moffit et al. (57). Data are presented from 3 independent replicates.

Supplementary Figure 2 Pan-Cytokeratin staining of Panc1 and Panc89 tumors and cyst formation in Panc89 Paraclone tumors. SCID beige mice were inoculated intrasplenically with either 1×10^4 Panc1 Holo- or Paraclone cells or Panc89 Holo- or Paraclone cells (10 mice/group). Resected tissues and tumor lesions were stained for **A)** Pan-Cytokeratin. Scale bars in the representative images: left = 500 μm ; right = 100 μm . **B)** Representative images of cyst formation in two different Panc89 Paraclone tumors. Scale bar = 200 μm .

Supplementary figure 3 Immunohistochemical analysis of L1CAM and Sox2 expression in Panc89 Holoclone tumors. SCID beige mice were inoculated intrasplenically with either 1×10^4 Panc89 Holoclone cells (10 mice/group). Extracted tumors were stained for L1CAM and SOX2. Data of the comparative analysis of L1CAM and SOX2 expression in all Panc89 Holoclone tumors (left) and of Panc89 Holoclone tumors exhibiting high L1CAM expression (right) are presented as median with range in violin plots $p < 0.033 = *$

A)



B)

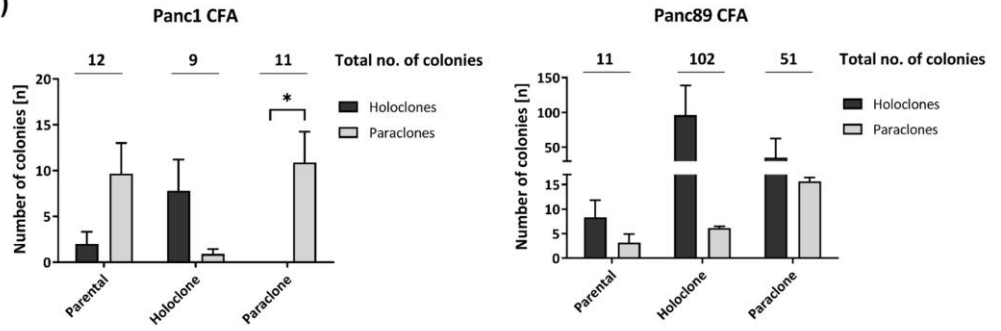


Figure 1

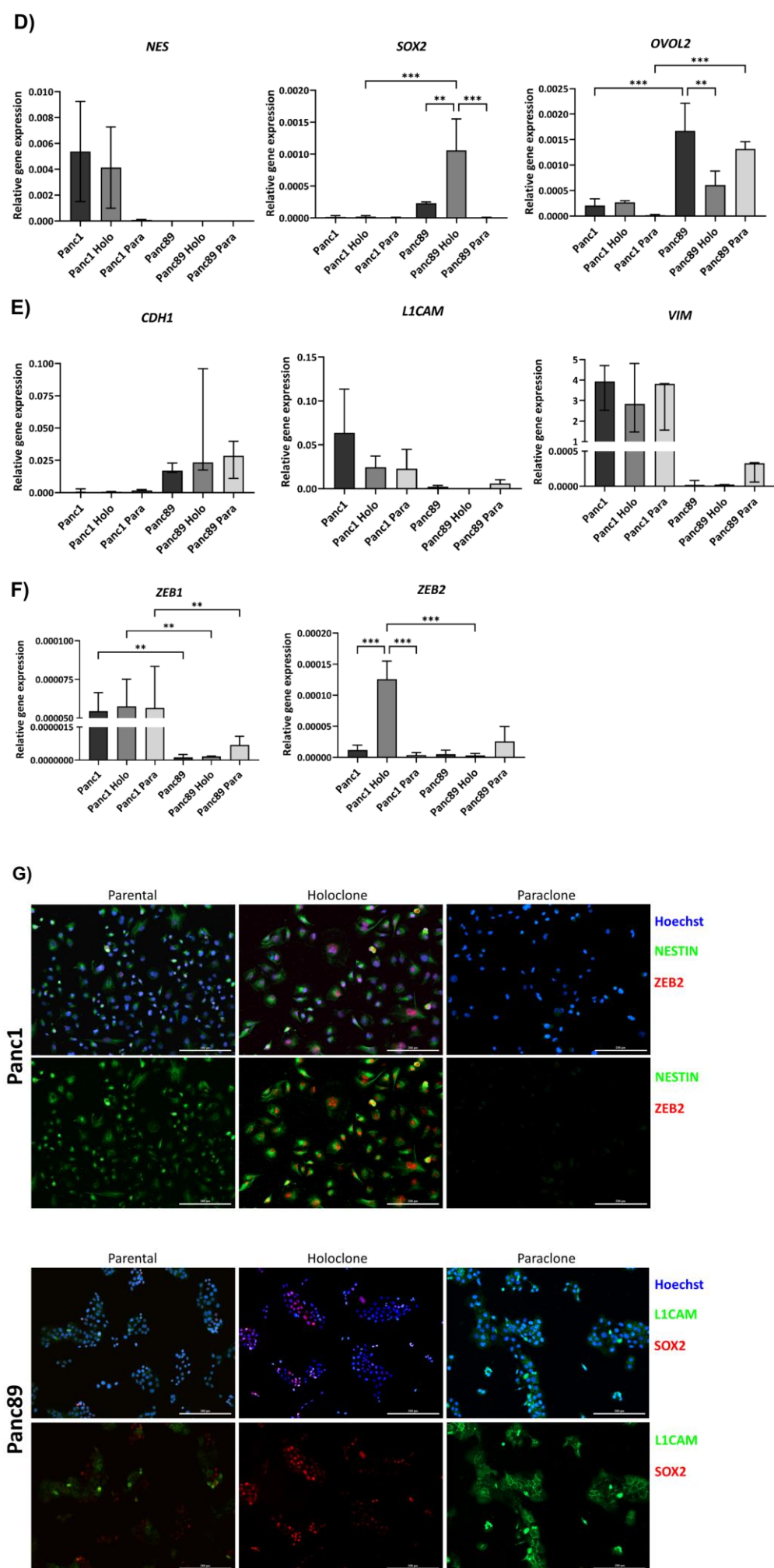
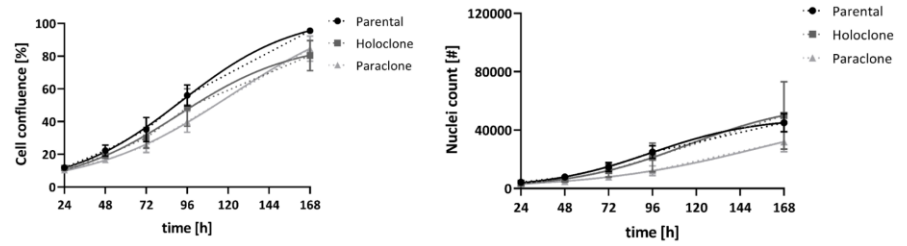


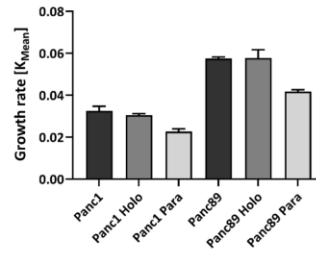
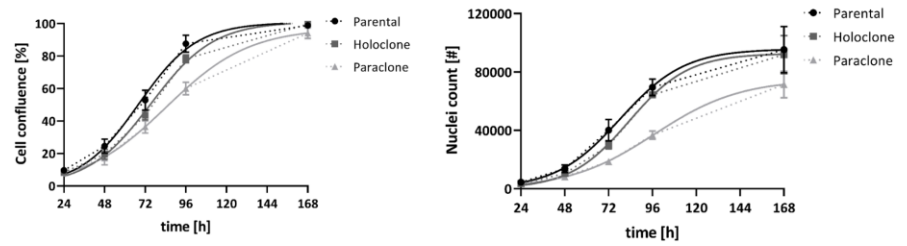
Figure 2

A)

Panc1

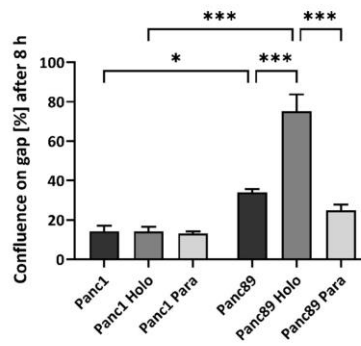


Panc89



B)

Migration ability



C)

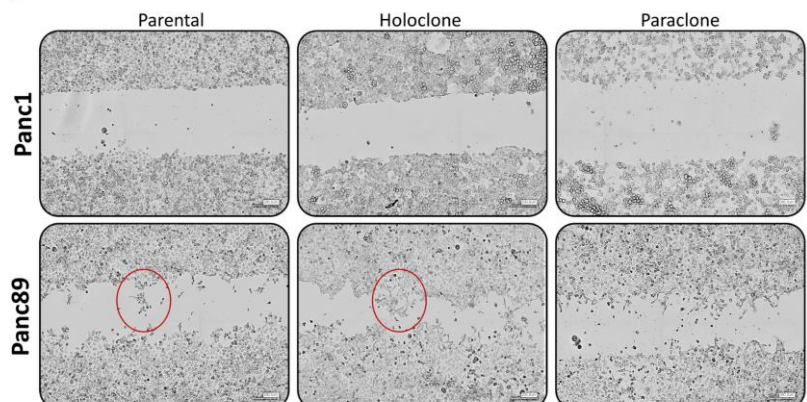


Figure 3

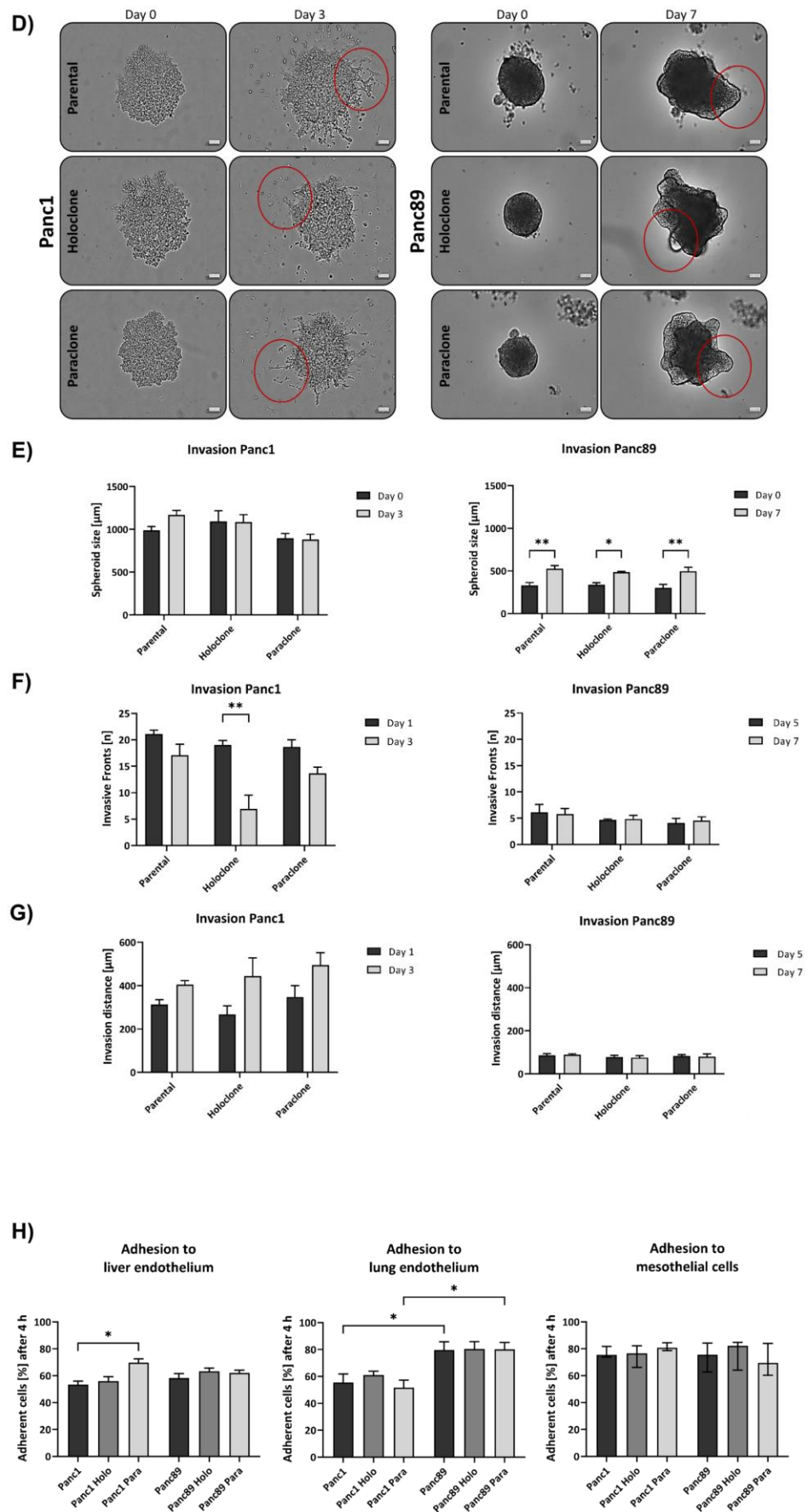
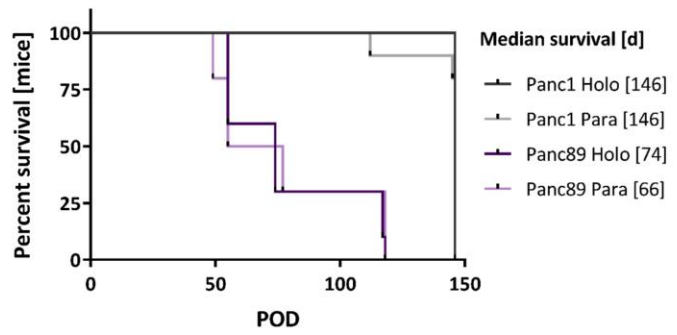


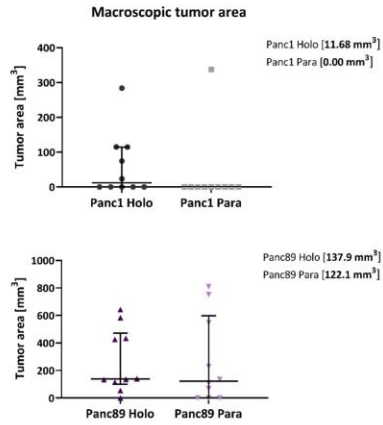
Figure 3

A)

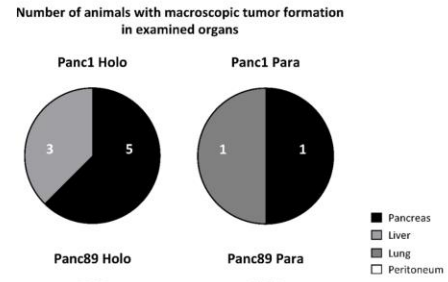
Kaplan-Meier-Survival



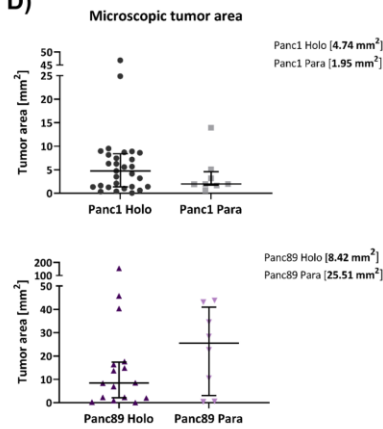
B)



C)



D)



E)

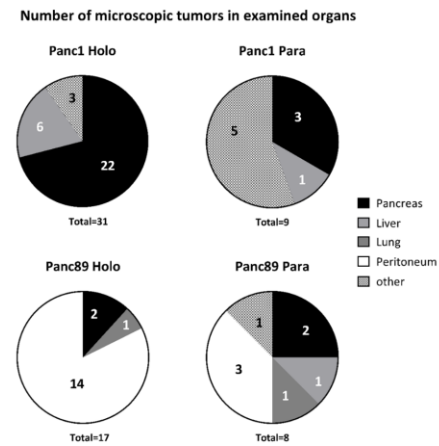


Figure 4

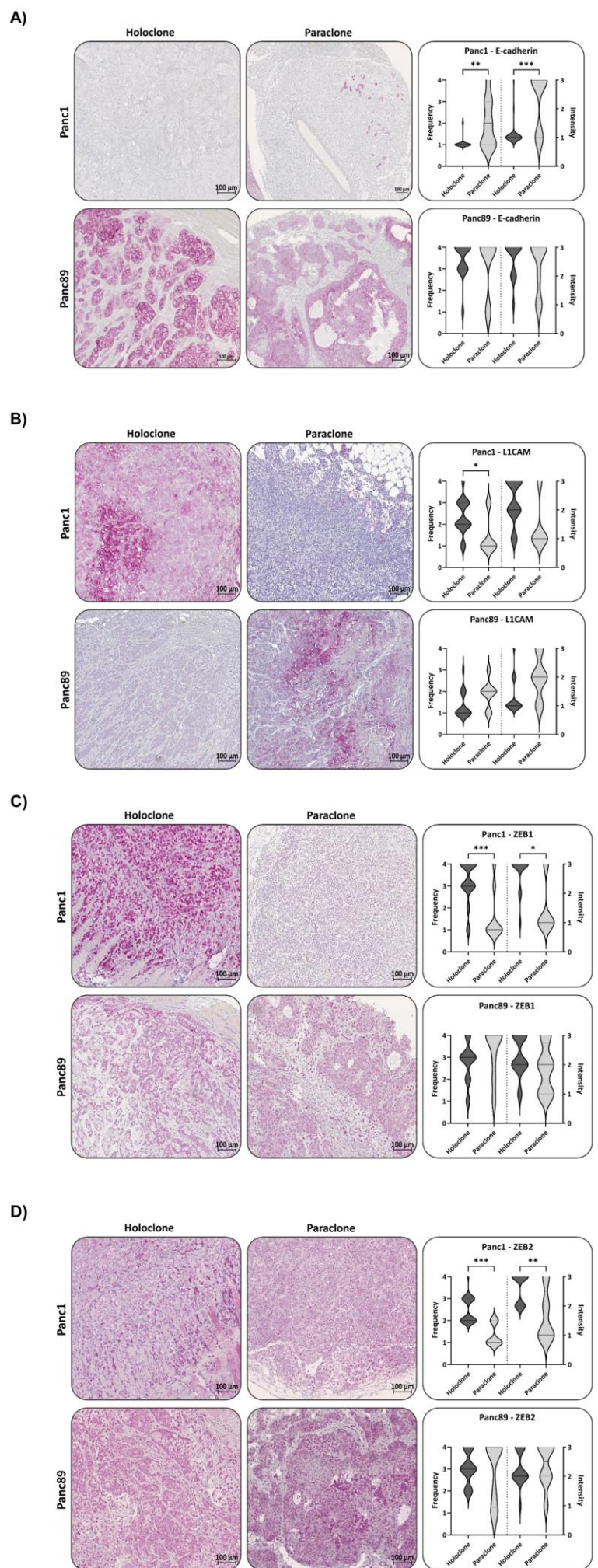


Figure 5

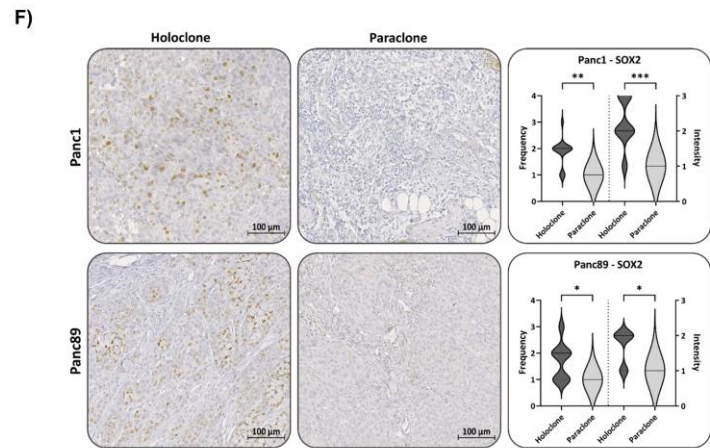
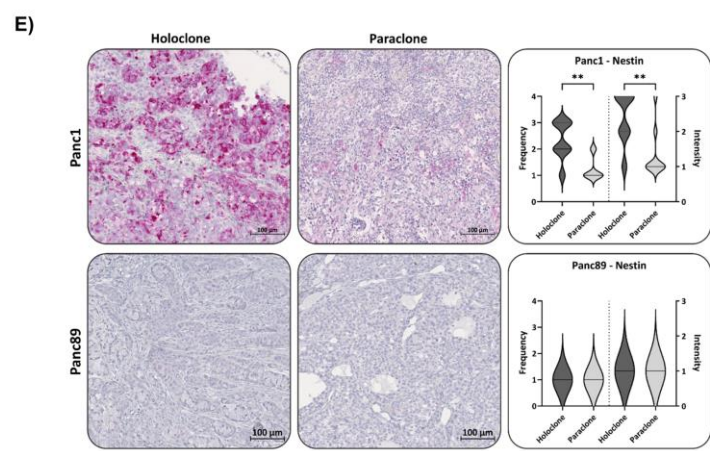
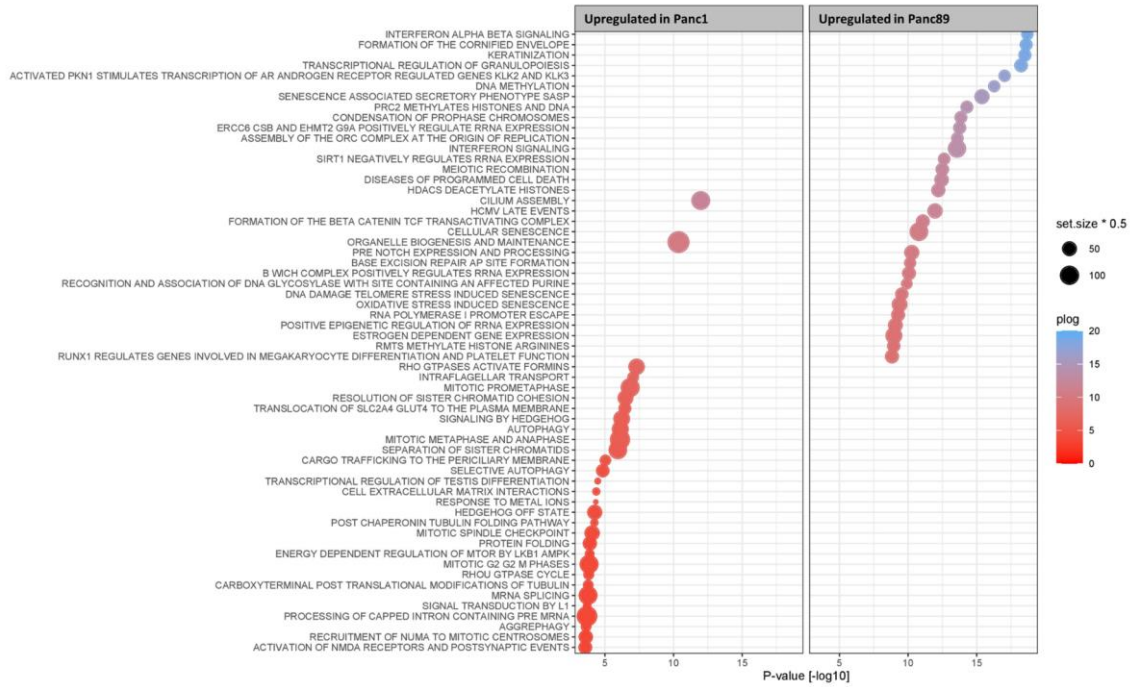


Figure 5

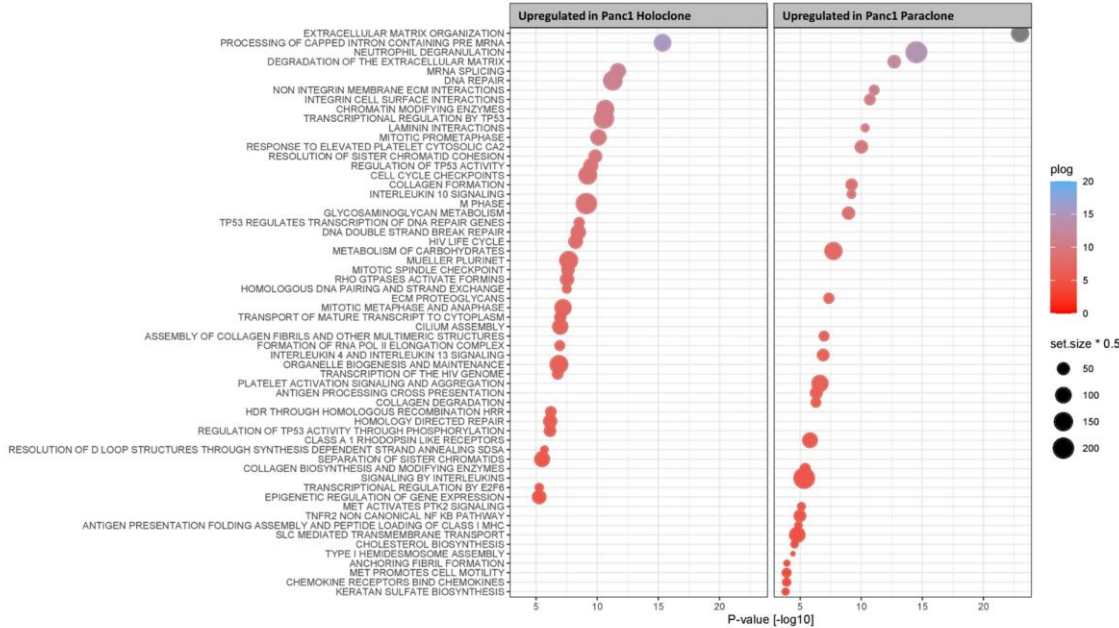
A)

Parental PDAC cells

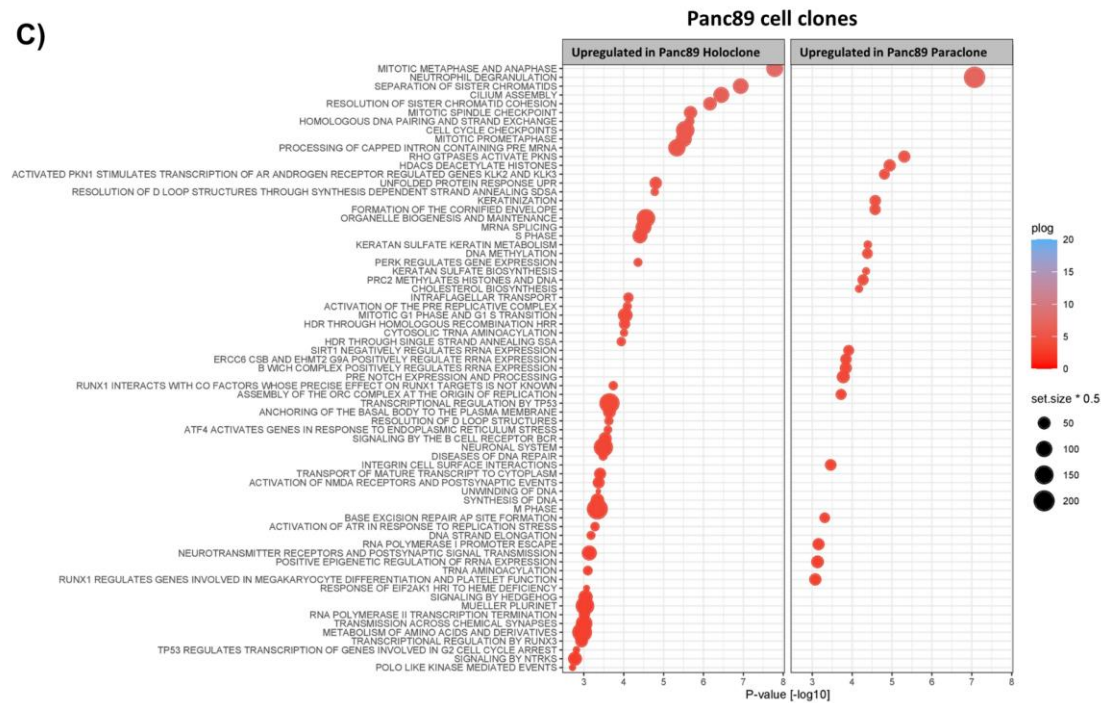


B)

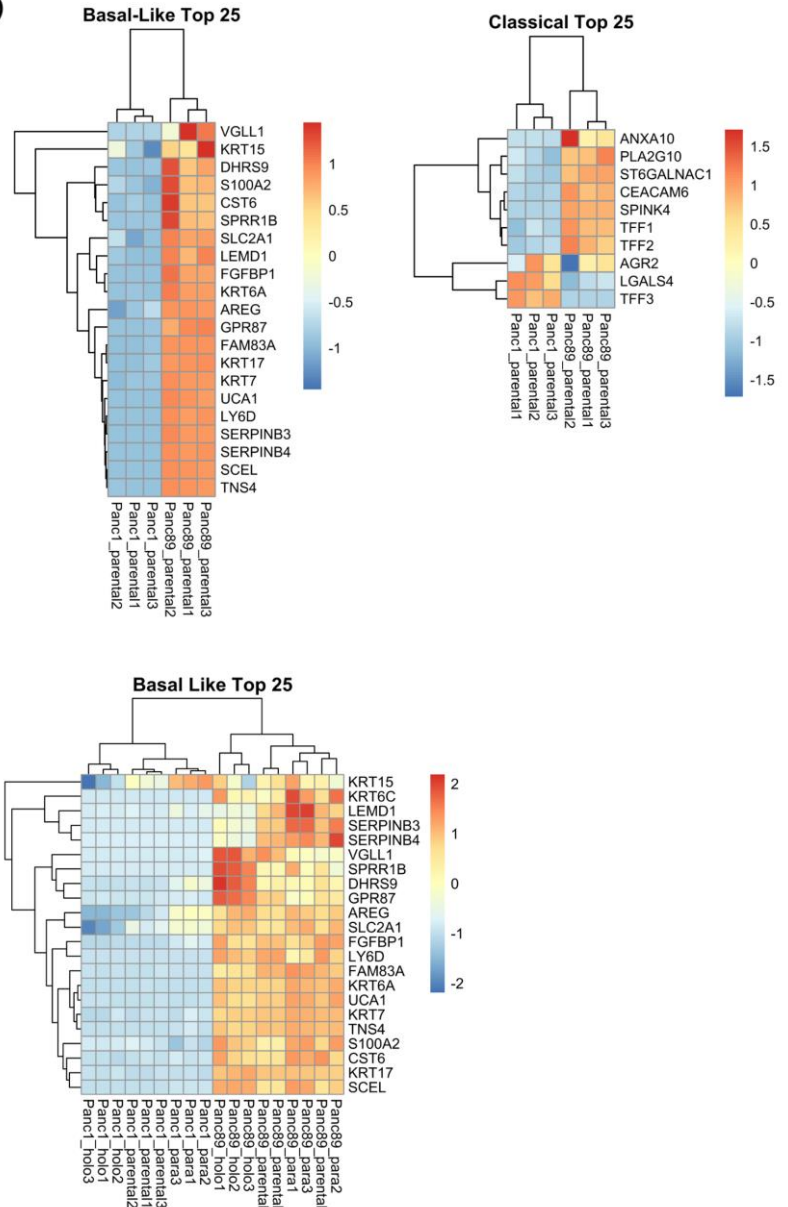
Panc1 cell clones



C)

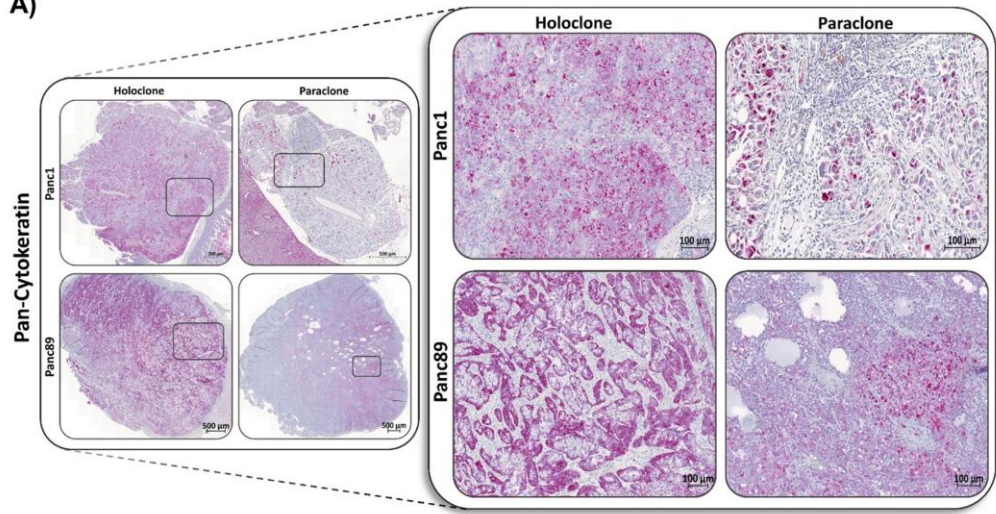


D)



Supplementary figure 1

A)



B)

Cyst formation in Panc89 Paraclone tumors

