Establishing murine models of biliary and pancreatic cancer for the rapid functional annotation of cancer genes

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aus Peine

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Establishing murine models of biliary and pancreatic cancer for the rapid functional annotation of cancer genes

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<tbody>
<tr>
<td>2-D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3-D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>AKT</td>
<td>Thymoma viral proto-oncogene</td>
</tr>
<tr>
<td>Alb</td>
<td>Albumin</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous-polyposis-coli protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CCA</td>
<td>Cholangiocarcinoma</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin dependent kinase inhibitor 2a</td>
</tr>
<tr>
<td>CDKN2B</td>
<td>Cyclin dependent kinase inhibitor 2b</td>
</tr>
<tr>
<td>CHC</td>
<td>Coll1a1 RMCE-based homing cassette</td>
</tr>
<tr>
<td>CK19</td>
<td>Cytokeratin 19</td>
</tr>
<tr>
<td>CK7</td>
<td>Cytokeratin7</td>
</tr>
<tr>
<td>CPT-11 (Irinotecan)</td>
<td>7-ethyl-10-[4-(1-pi-peridino)-1-piperidino</td>
</tr>
<tr>
<td>Cre</td>
<td>Cyclization recombinase</td>
</tr>
<tr>
<td>CRISPR/Cas9</td>
<td>Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>β-Catenin gene</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EpCAM</td>
<td>Epithelial cell adhesion molecule</td>
</tr>
<tr>
<td>ERBB</td>
<td>Epidermal growth factor receptor tyrosine kinase</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FZD</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GB</td>
<td>Gallbladder</td>
</tr>
<tr>
<td>GBC</td>
<td>Gallbladder Carcinoma</td>
</tr>
<tr>
<td>GEMM</td>
<td>Genetically engineered mouse model</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GTPases</td>
<td>Guanine-triphosphate-Hydrolase</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular Carcinoma</td>
</tr>
<tr>
<td>HDR</td>
<td>Homology directed repair</td>
</tr>
<tr>
<td>HEPPAR1</td>
<td>Hepatocyte paraffin-1</td>
</tr>
<tr>
<td>HNFα</td>
<td>Hepatocyte nuclear factor-4α</td>
</tr>
<tr>
<td>HTVI</td>
<td>Hydrodynamic tail vein injection</td>
</tr>
<tr>
<td>ICE</td>
<td>Inference of CRISPR Edits</td>
</tr>
<tr>
<td>INK4a/ARF</td>
<td>Inhibitor of cyclin dependent kinase 4a/ alternative reading frame</td>
</tr>
<tr>
<td>IPMN</td>
<td>Intraductal papillary mucinous neoplasm</td>
</tr>
<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma oncogene</td>
</tr>
<tr>
<td>LGR</td>
<td>Leucine-rich repeat containing G-proteil-coupled receptors</td>
</tr>
<tr>
<td>loxP</td>
<td>Locus of x-over</td>
</tr>
<tr>
<td>Lsl</td>
<td>Lox-stop-lox</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>Mitogen-activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>MCN</td>
<td>Mucinous cystic neoplasm</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>Mir31</td>
<td>MicroRNA31</td>
</tr>
<tr>
<td>mKate</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>MTAP</td>
<td>Methyl-thioadenosine phosphorylase</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>Myc</td>
<td>Myelocytomatosis oncogene</td>
</tr>
<tr>
<td>Nal-IRI</td>
<td>Nanoliposomal-Irinotecan</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch1 intracellular domain</td>
</tr>
<tr>
<td>PanIN</td>
<td>Pancreatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PDX</td>
<td>Patient-derived Xenografts</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol-3-kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-3,4,5 tri-phosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>Primary liver cancer</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma tumor suppressor protein</td>
</tr>
<tr>
<td>RMCE</td>
<td>Recombinase-mediated cassette exchange strategy</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNF43</td>
<td>Ring finger protein 43</td>
</tr>
<tr>
<td>rtTA3</td>
<td>Reverse tetracycline transactivator</td>
</tr>
<tr>
<td>sgCR8</td>
<td>sgRNA Chromosomal region 8</td>
</tr>
<tr>
<td>sgRNA</td>
<td>Single guide RNA</td>
</tr>
<tr>
<td>shRENNILLA</td>
<td>Control-shRNA</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SOX9</td>
<td>Sex determining region Y-box 9</td>
</tr>
<tr>
<td>Tet</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor suppressor 53</td>
</tr>
<tr>
<td>TRE</td>
<td>Tetracycline response element</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless int</td>
</tr>
<tr>
<td>wt</td>
<td>wildtype</td>
</tr>
</tbody>
</table>
2 Summary: Dissertation of Katharina Wolff

Establishing murine models of biliary and pancreatic cancer for the rapid functional annotation of cancer genes

Gallbladder cancer (GBC), cholangiocarcinoma (CCA) and pancreatic ductal adenocarcinoma (PDAC) have a five-year survival rate below 10%, as a result of late diagnosis, missing therapeutic options due to the genetic heterogeneity of the cancer types and sparse knowledge of the effects of specific genetic changes on tumor development. These cancers have a strong desmoplastic stroma reaction, which might interfere with therapeutic efficiency. For all three, accurate in vivo models will be elemental to display rapid and flexible genetic combinations and their pathological phenotype.

Murine liver and gallbladder cells can be cultured 3-dimensional (3D) as organoids and genetically manipulated via RNAinterference, CRISPR/Cas9 technology and overexpression of oncogenes. In vitro, organoids of the liver and gallbladder both show a biliary phenotype and are almost completely epithelial cell adhesion molecule (EpCAM) expressing cells. They grow out of a digested liver or gallbladder cell-suspension. EpCAM positive cells are found in less than 1% of liver cells, and in 21.1% of cells in the gallbladder.

Liver organoids with endogenous activation of mutated Kras	extsuperscript{G12D} and loss of floxed p53 due to Cre-recombinase activity grew faster in vitro compared to their wildtype counterpart. These organoids form with complete penetrance moderately differentiated adenocarcinomas when implanted subcutaneously into mice. Histologically, the tumors show Cytokeratin 19 positive ductal structures with a desmoplastic stroma, a hallmark characteristic of cholangiocarcinoma. For further analyses, cell lines of the resulting tumors were established and cultured either in 2D or 3D conditions (called tumoroids). Comparing the transcriptome profile of the unmodified wildtype organoids, the pre-injection mutational activated organoids and the related tumoroids in unsupervised cluster analysis, it showed a high similarity between the wildtype and pre-injected ones. In contrast, tumoroids have a markedly different expression profile. Differences were detected in the gene sets after mutational activation (Kras	extsuperscript{G12D/wt}; p53\textsuperscript{Δ/Δ}) in genes regulating the cell cycle, E2F and myelocytomatosis oncogene Myc, underlining the shown increased proliferation in vitro. In premalignant organoids growing in vivo, gene sets associated with inflammation and paracrine signaling became activated. Genomic copy number analysis also showed increased deletions and amplifications after Kras- activation and p53 deletion and even more after tumorigenesis in vivo, in regions often altered in human CCA. Serially transplanted 2D and 3D tumor cell lines still form moderately differentiated CCA with comparable histology. In vitro, MAPK Inhibitor selumetinib and PI3K inhibitor BKM120 act independently of the culture conditions and decrease cell growth.

Knockdown of the commonly mutated tumor suppressor gene in CCA, phosphatase and tensin homolog (PTEN), via RNAinterference leads to acceleration in tumor growth without
histological changes. To test the system in a more physiological environment, organoids transplanted intrahepatically develop similar CCAs. Additionally, comparable tumors arise when the CRISPR/Cas9 system targeting \( p53 \) and \( Pten \) is used in syngeneic \( Kras^{G12D/wt} \) organoids instead of shPTEN and excised \( p53 \) for the injection into immunocompetent mice. In these, first-line chemotherapy in CCA, Gemcitabine leads to a moderate survival benefit of 32 days compared to 42 days, but no complete tumor regression, reflecting the patient’s response.

Overexpression of MYC coupled with a red fluorescent protein mCherry in C57BL/6J organoids in combination with a GFP-coupled shRNA targeting \( p53 \) and a sgRNA altering adenomatous polyposis coli (Apc) transplanted into mice form tumors with different histology. The substitution of the oncogene KRAS with MYC leads to tumor-growth in a solid pattern in nests resembling the histology of hepatocellular carcinoma (HCC). Supportively, the transcriptome profiles of the tumoroids from the organoid-based murine CCA and HCC closely reflects the profile of authentic human cancers in comparative analysis.

The next aim was to transfer the experimental set up used for establishing the CCA mouse model to establish a gallbladder cancer model. \( Kras^{G12D} \) and \( sgp53 \) gallbladder organoids transplanted into mice form gallbladder adenocarcinomas with accelerated growth when \( Pten \)-expression also gets interrupted. In demonstrating the plasticity of these organoids, changing the oncogene to overexpression of mutated epidermal growth factor receptor 2 (ERBB2) leads to papillary gallbladder cancer. The liposomal formulation of irinotecan called Nal-IRI does lead to a therapeutic benefit compared to irinotecan, probably due to improved drug delivery to the tumor site.

In the KRAS-driven pancreatic embryonic stem cell (ESC) based murine model, the role of loss-of-RNF43, as well as large homozygous deletions, were addressed. Multi allelic-ESCs were targeted with regulatable CRISPR/Cas9 constructs for an organ-specific and inducible genome editing. Loss of RNF43 led to reduced survival and increased tumor development.

Organoid cultures have unique opportunities and plasticity to model cancer. In both primary liver cancer and gallbladder cancer, they stay in an untransformed stage even after serially passages, can be cryoconserved, unlimitedly expanded and genetically modified according to cancer-type specific needs of investigations. Carcinogenesis can be studied starting from genetically wildtype cells with stepwise tumor-development and their influence on the therapy response in combination with the expression of a fluorescent protein, which makes the system traceable. This model and the developed ESC-based genetically engineered pancreatic mouse model have the advantage of displaying tumors resembling the human disease in an immunocompetent environment. In contrast, the ESC-based model keeps due to its inducible and temporal control the advantage of giving rise to tumors endogenously in a healthy environment. The CRISPR/Cas9 technology, with its possibility of easy and fast introduction of all kinds of genetic changes, demonstrates high efficiency in both models.
3 Zusammenfassung: Dissertation von Katharina Wolff

Etablierung eines murinen Models für Tumore des biliären Systems und des Pankreas und dessen schnelle und funktionelle Annotation von Tumor-Genen

Gallenblasenkrebs (GBC), Cholangiokarzinom (CCA) und duktales Adenokarzinom des Pankreas (PDAC) haben eine 5-Jahres-Überlebensrate von unter 10% aufgrund der häufig erst späten Diagnose, fehlender Therapiemöglichkeiten durch die große Heterogenität der Tumorarten und das geringe Wissen über die Auswirkungen von spezifischen, genetischen Veränderungen auf die Tumorentwicklung. Diese Tumorarten haben die Eigenschaft, ein desmoplastisches Stroma auszubilden welches dann die therapeutische Effizienz beeinflussen kann. Sowohl für GBC, CCA als auch PDAC sind in vivo Modelle dringend notwendig und grundlegend für weitere Forschung. Diese Modelle sollten akkurat den pathologischen Phänotyp schnell und flexibel mit unterschiedlichen genetischen Kombination abbilden können.


Ein Vergleich des Transkriptomprofils der unmodifizierten Wildtyp-Organoiden und der genetisch manipulierten, pre-injizierten Organoiden und ihre dazugehörigen Tumoroide zeigen in nicht-überwachten Klusteranalysen eine große Ähnlichkeit zwischen den Wildtyp- und den pre-injizierten Organoiden. Das Profil der Tumoroide unterscheidet sich deutlich von beiden anderen. Im Vergleich der Expression bestimmter Gengruppen zeigt sich, dass nach der Aktivierung von Kras\textsuperscript{G12D} und der Deletion von p53 Gengruppen welche den Zellzyklus regulieren, sowie der E2F-Signalweg und Gene aus dem Signalweg des Onkogens


Ein Austausch des Onkogens KRAS durch ein überexprimiertes MYC in Verbindung mit einem rot fluoreszierend Protein mCherry in C57BL/6J Organoiden und zusätzlich exprimierter GFP-gekoppelter shRNA gegen p53 und sgRNA welche gegen das Gen Adenomatous Polyposis Coli (Apc) gerichtet ist, führt transplantiert in Mäuse zu Tumoren mit einer komplett anderen Histologie. Die Tumorzellen wachsen in einer soliden Struktur in Form von Nestern ähnlich der Histologie von Hepatozellulären Karzinomen (HCC). Unterstützend zu den histologischen Ähnlichkeiten sind die Transkriptomprofile der Tumoroide etabliert aus den organoid-basierten murinen CCA und HCC sehr ähnlich zu den Profilen von authentischen, humanen Tumoren.

Der experimentelle Aufbau den wir für die Etablierung des CCA-Maus Models genutzt haben wurde auf Gallenblasen-Organoiden und die Entwicklung eines Gallenblasetumor-Mausmodels übertragen. Die in Mäuse transplantierten Kras\textsuperscript{G12D/wt}, sgp53

In dem murinen Model basierend auf embryonale Stammzellen (ESCs) mit einem organspezifisch induzierbaren, latenten Kras\textsuperscript{G12D} wurden die Rolle des RNF43-Verlustes und große homozygote Deletionen im Pankreas betrachtet. Multi-Allel ESCs enthalten regulierbare CRISPR/Cas9 Konstrukte welche Pankreas-spezifisch und Doxycylin-induzierbar das Genom verändern. Der Verlust von RNF43 führt zu einem reduzierten Überleben und erhöhter Tumorentwicklung.

4 Introduction

Liver, gallbladder (GB), bile ducts and pancreas arise from the same embryologic origin and share similarities in function and morphology. Collectively, they control the metabolism of the organism, support digestion by secreting either enzymes (pancreas) or solubilizing factors (bile from the liver) into the intestine through the same duct (hepatopancreatic duct). Adult pancreas, liver, and gallbladder share typically epithelial cells forming a ductal network with direct contact of secretory cells to the ductal network. The global incidence of cancer of these tissues is increasing rapidly, whereas more than 95% are carcinomas with adenocarcinoma as the most common histological type. Next to their similar morphology, prognoses are extremely poor. Reasons for that are late diagnosis and little knowledge about the diseases. These gastrointestinal tumors are genetically a very heterogeneous group, making it difficult to study them in vitro, and there is limited availability of appropriate mouse models. Inflammations of these organs lead to a higher risk of cancer, whereas the stem cell biology and regeneration capacity is an important research field.

The second problem of primary liver cancer (PLC) pancreatic ductal adenocarcinoma (PDAC) and gallbladder carcinoma (GBC) is the missing, potent treatment options owed to the late diagnosis and heterogeneity of the tumors, inter- and intratumoral. Still, the primary option is surgery, which is mostly not possible due to the advanced stage cancer at the time of diagnosis, leaving chemotherapy treatment as an only adjuvant therapy option. However, chemotherapy treatment is frequently ineffective by targeting only specific pathways, which might be escaped by cancer, leading to chemo-resistance. Therefore, personalized therapy might be a solution, for which profound understanding of the genetic landscape of all tumor types and their classification is required.

Briefly, a short overview of the cancer types is given, followed by a description of the genomic landscape and a summary of available models to study these cancer types.

4.1 Primary liver cancer: Hepatocellular carcinoma and Cholangiocarcinoma

PLC is the fourth most common cancer-related cause of death worldwide, even though it is only the sixth most frequent form of cancer. Hence, fatality and increasing rates of incidence between 1990 and 2015 by 75% make a better understanding of tumor development essential. Reasons for the high mortality are generally late diagnosis and ineffective treatments. Detection of liver cancer is often coincidental, and the further diagnosis for the specific subtypes is
complex. There are certain blood biomarkers, like conventional liver functional markers or serum tumor markers, but mainly imaging techniques are used for diagnosis.\(^6\)

The most common subtype of all primary liver cancers is hepatocellular carcinoma (HCC). Strong risk factors are Hepatitis virus B and C infections, liver damage due to aflatoxin or alcohol and certain other metabolic liver diseases. Very often, lifestyle and environmental factors determine the fate of HCC, but also genetic predispositions increase the risk. HCCs have a hepatocytic-phenotype and grow in a certain pattern – tumor cells grow nest-forming without any desmoplastic stroma.\(^7\)

Current standard therapy for HCC is the drug Sorafenib (inhibition of the tyrosine kinase Raf and others) but provides only little prolongation of the patient survival. Other recently approved therapeutic targets are: Regorafenib (stronger inhibition of a broader range of tyrosine kinase and angiogenesis\(^8\)) and Nivolumab (inhibitor of the immune checkpoint inhibitor programmed cell death protein-1 PD-1\(^9\)).

Intrahepatic Cholangiocarcinoma (CCA) is the second common subtype of all primary liver cancers, together with HCC accounting for around 99% of all liver cancers.\(^10\) HCC and CCA are not always separately defined, therefore, HCC-CCA mixed subtypes occur.

CCAs are aggressive tumors that are mostly detected when they are already in an advanced stage. Pain is not a common symptom but is rather unspecific like jaundice, weight loss, abdominal discomfort, malaise, pruritus and pale stool and is typically absent in the early stage of the disease. Patients developing CCA are, on average, over 70 years old and more likely, male.\(^10\) CCAs show a marker of cholangiocyte differentiation arising from the epithelium of the biliary tree with a strong desmoplastic reaction.\(^11\) Most commonly, they are classified based on their anatomical location: intrahepatic, perihilar and distal CCA. The most frequent one is intrahepatic, where in this work, following the short form, CCA refers to intrahepatic CCA. Other classifications, for example, due to different patterns of growth or histological characteristics consider more pathological characteristics, which would be useful concerning clinical treatment, but is not regularly used at present.\(^12\)

Like in HCC, risk factors for CCA are linked to infections or metabolic diseases in which cell death, increased cell proliferation and chronic inflammation play a role (cholangitis, primary sclerosing cholangitis, liver flukes and several more). Also, environmental factors like nitrosamin in food, dioxins, vinyl chlorides or heavy smoking and alcohol consumption might be significant factors in CCA development but in a smaller concern than for HCC. CCA, in general, occurs \textit{de novo} due to genetic alterations.\(^{5,11,12}\) CCAs are a diverse group by cause of growing pattern, histological features, and genetic context inter- and intra-tumoral. This
heterogeneity is supported by increasing sequencing-data from human tumor specimens identifying various genetic and transcriptomic alterations from which the molecular effects on the tumor development remain unclear. This expression profile is distinct between HCCs and CCAs.\textsuperscript{13–15}

CCAs often present with multiple liver lesions, large size tumors or regional lymph node involvement resulting in median free survival of 12-36 months. The currently available chemotherapeutics and targeted therapies have only little effect on the survival of the patients.\textsuperscript{16} First-line chemotherapy is the combination of gemcitabine and cisplatin. Gemcitabine (2',2'-difluoro-2'-deoxycytidine, dFdC) appears in the organism phosphorylated to a diphosphate or triphosphate derivate which then binds the DNA, incorporates one single nucleotide and blocks the DNA polymerase processing.\textsuperscript{17} Besides, it interferes with the enzyme ribonucleotide leading to a depletion of deoxycytidine-triphosphate (dCTP) and supports the blockage of the DNA replication. Gemcitabine is, therefore, an effective anticancer agent.\textsuperscript{18}

Now available genomic analyses led to the invention of molecular targeted therapies like receptor-tyrosine-kinase inhibitors, therapeutics targeting epigenetic alterations and also immunotherapy.\textsuperscript{3}

\section*{4.2 Gallbladder Carcinoma - the most aggressive malignancy of the biliary tract}

The most aggressive malignancy of the biliary tract is gallbladder carcinoma (GBC)\textsuperscript{19} ranked sixth most common gastrointestinal tract cancer. Fortunately, amongst all cancer types, it is a rare neoplasm but with a very high mortality rate also because of its immense metastatic potential. Worldwide, the median survival range is between 5.7 months to 15.5 months.\textsuperscript{20,21}

Main risk factors besides genetic predisposition and demographic factors include chronic cholecystitis, gallstones, dietary factors, chronic gallbladder infections and exposure to specific chemicals in the environment.\textsuperscript{22} Like pancreatic and liver cancer, GBC also progresses mostly asymptomatic\textsuperscript{23} and is mainly diagnosed at routine cholecystectomy or surgery. The most common symptom is right upper quadrant pain, which only appears when the cancer is already advanced.\textsuperscript{24} Otherwise commonly used diagnostic options are imaging with ultrasonography, computed tomography, magnetic resonance imaging and magnetic resonance cholangiopancreatography.\textsuperscript{25}

First-line chemotherapeutic treatment is the same as for CCA, gemcitabine and cisplatin combinatorial treatment. The comparably low survival rate of 11.7 months with combinatorial
treatment and the fact that currently, no second-line treatment is available, underlines the urgent need of new therapeutic options especially in GBC.\textsuperscript{26}

Irinotecan (CPT-11) is a prodrug converted by carboxylesterase into the 100-1000-fold more active metabolite SN-38. SN-38 inhibits the Topoisomerase I, which is active in the nucleus to relax strained torsional DNA. Inhibiting Topoisomerase I has an antitumoral effect \textit{in vitro} and \textit{in vivo}.\textsuperscript{27} Due to strong side effects and short half-life by direct drug administration, a liposomal-delivery system is used for CPT-11 delivery. Encapsulated CPT-11 into long-circulating liposome-cased particles (called Nanoliposomal-Irinotecan Nal-IRI) improves drug stability and increases the drug load at the tumor site. Compared to CPT-11, Nal-IRI has shown greater efficacy in colon-cancer xenograft mouse models\textsuperscript{28} and is already in phase II studies for metastatic pancreatic cancer.\textsuperscript{29} Therefore, Nal-IRI might be a promising drug in GBC treatment.

\subsection*{4.3 Pancreatic cancer – lowest survival rate of all cancer types}

The five-year survival rate of pancreatic ductal adenocarcinoma (PDAC) is 8\%, the lowest of all cancer types. In contrast to most others, the survival rate did not increase within the last years.\textsuperscript{30} Resection remains the main treatment-method, whereas even though the surgical treatment has improved, it did not raise the survival time. Current standard care is surgical resection followed by neoadjuvant treatment with gemcitabine or a combinatorial treatment. Recently, FOLFIRINOX has shown to increase the median-disease-free survival to 21.6 months compared to 12.8 months with gemcitabine only treatment.\textsuperscript{4}

PDAC arise out of precursor lesions of different types: Pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN).\textsuperscript{31} MCN is almost exclusively observed in women and a rare disease.\textsuperscript{32} IPMNs and PanINs have some overlapping and some distinct genetic alterations with each other, and both grow in intraluminal mucin and papillary pattern differentiated in size. PanINs are microscopic lesions smaller than 5 mm and IPMNs are bigger than 10 mm, grossly visible. The mechanism of development of either of the precursor lesions is not well-known and IPMNs might develop from low-grade PanINs or independently.\textsuperscript{33}

\subsection*{4.4 Genetic landscape of PLC, BTC and PDAC is diverse}

The most mutated genes in PDAC, GBC, and CCA are the Kirsten rat sarcoma oncogene \textit{KRAS} and the tumor suppressor \textit{TP53} (Figure 1). Yet, they all have distinct heterogeneous genotypes designating their specific phenotype, diagnosis, prognostic significance, and espe-
cially the need for specific or even personalized therapy.\textsuperscript{34} The mutation rate of CCA and PDAC is comparable.\textsuperscript{13} All three cancers are not only heterogeneous among patients, also many tumors show a significant intra-tumoral heterogeneity with signs of tumor-evolution in terms of morphology, genome composition, and mutated genes. This chapter will give a short overview of the genetic landscape of the previously described gastrointestinal cancers and some detailed genes focused on in this work. Figure 1 shows the heterogenetic landscape important in this work with its similarities and differences between the tumor subtypes.

![Gene Mutations Table]

<table>
<thead>
<tr>
<th>Gene</th>
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Figure 1: Genetic Alterations in most common gastroenterology-cancers. Numbers show frequency in %. Data from www.chiportal.org for all available studies for each cancer type in November 2019. The table shows nicely the overlapping frequency of signaling pathways in all cancer types with individual specifications. Red color marks genes related to the MAPK-pathway, orange of the PI3K-pathway, green of the WNT-signaling and blue genes of the INK4A/ARF locus. On top 4x magnification of hematoxylin- and eosin-stained examples of a tumor-histology.

In a study of 150 PDAC-patients, the neoplastic cellularity had a median of 18%, with a range between 0 – 53%, pathologically reviewed. Determining the neoplastic cellularity with whole exome sequencing, it ranged from 9-89% with a median of 33%. This shows adequate in
numbers the high amount of desmoplastic stroma, characteristic for PDACs but making genome sequencing difficult.\textsuperscript{35}

Genetic changes in HCC are only a small part of the risk factors in the path to cancer. Therefore, some few mutations are very common: The telomerase reverse transcriptase (\textit{TERT}) promoter (60\% mutation rate), Tumor suppressor 53 \textit{TP53} (35-50\%) and \textit{CTNNB1} (gene expressing $\beta$-\textit{Catenin}) (19-40\%). The following most frequently mutated genes in HCC are mutated with a rate of approximately or below 10\% (e.g. \textit{AXIN1}, \textit{ARID1A}, \textit{WWP1}). The tumor suppressor phosphatase and tensin homolog (\textit{PTEN}) is mutated in only 3\% of HCCs.\textsuperscript{36} In HCC, also microRNA expression and epigenetic factors may play an important role. Mutation-specific subtypes in HCC are not evident, but in contrast showed stable molecular subtypes of HCC (based on stemness gene expression patterns) and CCA (linked to proliferation subtype and inflammation subtype).\textsuperscript{37}

Depending on clustering of mutation, copy number, gene expression and epigenetic data, CCA can be divided into molecular subtypes independent from the anatomical position.\textsuperscript{38} Inactivating mutations were found in tumor suppressor genes \textit{ARID1A}, \textit{ARID1B}, \textit{BAP1}, \textit{PBRM1}, \textit{TP53}, \textit{STK11}, \textit{PTEN} and gain-of-function mutations in oncogenes like \textit{IDH1}, \textit{IDH2}, \textit{KRAS}, \textit{BRAF} and \textit{PIK3CA}. \textit{KRAS} is one of the most frequently mutated genes, but its mutation frequency varies between 20-50\% depending on the study.\textsuperscript{13,38} These differences and also the huge amount of data available now due to sequencing studies makes it even more important to study the molecular effects of the epigenetic and genetic changes detected to validate the genomic data.\textsuperscript{38} The most common altered signaling pathways are the Ras and Phosphoinositid-3-kinase (\textit{PI3K})-\textit{, p53/cell cycle-} and \textit{Tgf-\beta/Smad4} pathways. In a cohort of 72 CCAs, all showed a mutational change in the Mitogen-activated protein kinase (\textit{MAPK}) pathway, mostly \textit{KRAS} and \textit{PTEN} in CCA compared to RPSK6KA3 in HCC. 45 of these CCAs have a mutation in the \textit{p53/cell cycle signaling}, which is also very common in HCC.\textsuperscript{39} Epidermal growth factor receptor tyrosine kinase 2 (\textit{ERBB2}) in CCA is amplified in 3.9 – 8.5\%, plus nine different occurring activating mutations in the gene of \textit{ERBB2} occur with a frequency of 2\%.\textsuperscript{38}

Another common class of mutation is the somatic structural rearrangement causing gene disruption, for example, deletions, rearrangements or gene activation via copy number gain or amplification or the fusion of two genes building a novel oncogenic gene product. 50 regions of recurrent gain were identified in CCA (oncogenes like \textit{MET}; \textit{NOTCH1} and \textit{GATA6}) and 73 regions of loss, including the tumor suppressor genes \textit{CDKN2A}, \textit{SMAD4}, \textit{TP53}, \textit{BRCA1}, \textit{ARID1A}, \textit{PBRM1} and \textit{SMARCA4}.\textsuperscript{40}
Whole genome sequencing of GBC revealed 3 or more nonsynonymous somatic mutations in 23 genes. Targeted sequencing showed genes frequently mutated in cancer also significantly altered in GBC, $TP53$ (47.1%), $KRAS$ (7.8%) and $ERBB3$ (11.8%) and $ERBB2$ is mutated in 9.8% of GBC. A different whole exome sequencing study with a bigger cohort showed comparable results ($TP53$ 27%, $KMT2C$ 11%, $SMAD4$ 11%, $ERBB2$ 7% and $ERBB3$ 8% mutated), but found in addition $KMT2C$ and $SMAD4$ as frequently mutated. $ERBB2$ overexpression was described previously in human and murine GBC progression. Mutations were also found in $EGFR$ and $ERBB4$ with both 3.9% frequency. Patients with altered ERBB-pathway are associated with poor prognosis in GBC.

Whole exome sequencing of PDAC revealed somatic DNA alterations like single nucleotide variants, small insertions and deletions and somatic copy number alterations. Profiling of PDAC showed 93% of all PDACs having a $KRAS$ mutation and in the last 7% of wildtype KRAS expressing tumors 60% harbor an alternative RAS/MAPK pathway activating alterations. This highlights the importance of this pathway, for more details see 4.4.2. Additional mutations in tumor suppressors or oncogenes are needed for the progression of KRAS-driven precursor lesions into PDAC, most frequently additional acquired mutations are within the genes cyclin dependent kinase inhibitor 2a ($CDKN2A$), $TP53$ and $SMAD4$. This inactivation occurs at rates over 50%, followed by a handful of genes mutated with a frequency of 10%. Significant recurrent mutations are also $RNF43$, $ARID1A$, $TGFßR2$, $GNAS$, $RREB1$ and $PBRM1$ and the long list of infrequently mutated genes results in a high inter-tumoral heterogeneity. Bailey et. al separates genetic alterations into different groups: Next to the activating mechanisms of KRAS, 78% of all genes are part of the G1/S checkpoint machinery, 47% play a role in TGF-ß signaling, 24% in histone modification, 14% build the SWI/SNF complex, 5-12%, 5% in WNT signaling defects and 16% RNA processing.

### 4.4.1 $TP53$, the mutual mutated tumor suppressor gene

$TP53$ encodes for $p53$, a tumor suppressor with multiple functions. $TP53$ is the most commonly mutated human gene, playing a role in over 50% of all human cancers (including PDACs, BTCs, CCAs and HCCs) correlated with worse patient-survival. In addition, p53-pathway regulating genes like murine double minute 2 ($MDM2$) can be altered. $p53$ is a transcription factor regulating more than 2500 genes that impact highly diverse cellular processes. Under physiological conditions, $p53$ blocks cancer by enabling DNA repair mechanisms or advancing cellular death programs. Upon cellular stress signals like DNA damage, $p53$ is activated and results in expression of its target genes leading to cell-cycle arrest, DNA repair or apoptosis but
might also lead to senescence or angiogenesis, depending on the intensity of the cellular stress. p53 as an attractive drug target did not lead to many effective therapies, mainly because of its huge complexities in p53-network or also their opposed activities.\textsuperscript{45,46}

\subsection{Two most frequently altered cancer related pathways: MAPK- and PI3K-mTOR pathway}

\textit{Ras} is a small protein of 188-189 amino acids of the guanine-triphosphate-hydrolase (GTPases) family and comprises. Kirsten-Ras (\textit{KRAS}), Harvey-Ras (\textit{HRAS}) and Neuroblastoma-Ras (\textit{NRAS})\textsuperscript{47} are RAS-family members and act as a binary molecular switch to communicate external signals to the nucleus. The majority of RAS mutations in CCA, BTC and PDAC occur in \textit{KRAS}, only a few in \textit{NRAS} or \textit{HRAS}. RAS or one of the related genes are in 30\% of all human cancers mutated.\textsuperscript{48}

\textit{KRAS} got identified with a great mutational heterogeneity, multiple different mutations in subclones were detected.\textsuperscript{35} Typically, in the development of PDAC, \textit{KRAS} is an early occurring mutation and alone results in the development of PanIN but does not have high malignant potential.\textsuperscript{49} Major mutations in \textit{KRAS} are at the \textit{KRAS} codon 12, G12D, G12V and G12R, less frequent are mutations at codon 13, 61 and 146. There might be an impact of the type of mutation of \textit{KRAS} on the patient’s survival, resistance to apoptosis and the metastatic efficiency.\textsuperscript{50} \textit{KRAS} mutation detected differ in the frequency, depending on the cohort and analysis: 16\% found in a cohort in Thailand and also 16.7\% as the second common genetic change in CCA in 103 patients of China. In contrast, \textit{KRAS} is not significantly high mutated in HCC genome sequencing projects.\textsuperscript{39}

The MAPK-pathway is activated by factors at a cell-surface receptor starting a cascade of kinases to transduce extracellular signals into cellular responses. Three enzymes are activated in series, the MAPK-kinase kinase MAPKKK, a MAPK kinase MAPKK and the MAPK itself. In mammals 14 MAPKKs, 7 MAPKKs and 12 MAPKs exists.\textsuperscript{51} MAPKs are tyrosine kinases activated by a diverse range of stimuli of cytokines, growth factors, neurotransmitters, hormones, cellular stress and cell adherence and transduce that signal into an appropriate physiological response including cellular proliferation, differentiation, development, inflammatory responses and apoptosis. Upstream of that MAPK-cascade is e.g. the G-protein RAS which gets activated by a receptor tyrosine kinase stimulated by an extracellular factor. RAS-induced cellular response acts mostly through the MAPKKK RAF, which then phosphorylates the MAPKK MEK1/2 which in turn phosphorylates the MAPK ERK1/2. Extracellular signal-regulated kinase ERK (also called P44/42) is the major effector of RAS GTPase and regulates
cell survival, proliferation and motility. There are other MAPK-pathways, stimulated through different factors but with intense crosstalk with each other and different pathways and the RAF/MEK/ERK pathway is the most often dysregulated one in cancer. Wildtype (wt) KRAS bearing PDACs often carry alterations in the mTOR pathway, making this an additional possible target for therapy of PDACs with wt-KRAS. Therefore, the other most important pathway in regulating cell fade in response to extracellular cues is the Phosphatidylinositol 3-Kinase-Mammalian Target of Rapamycin (PI3K-mTOR) pathway. The lipid kinase PI3K gets recruited to growth factor receptors, generates there Phosphatidyl inositol 3,4,5 tri-phosphate PIP3 which in turn recruits the protein kinase Thymoma viral proto-oncogene (AKT) to the plasma membrane. Subsequently, AKT is activated by 3-phosphoinositide-dependent-kinase 1 PDK1 and the mTOR complex 2 and in turn activates many factors regulating survival, proliferation and motility, for example, some intermediate steps the eukaryotic initiation factor 4E binding protein (EIF4EBP) and p70 ribosomal s6 kinase (S6K1), both regulation ribosome biogenesis and the protein-translation to promote cell growth and division. The lipid phosphatase PTEN negatively regulates the PI3K-signaling by dephosphorylation of PIP3 and maintaining low levels of PIP3 to keep AKT deactivated.

In both pathways, signaling intensity and duration needs to be strongly regulated by positive feedforward and negative feedback loops. Activators of the pathways overlap partially, whereas the degree of activation by specific growth factors depends on the number of growth factors and the expression and localization of cell surface receptors recognizing these agonists. Ras-MAPK and PI3K-mTOR1 pathways cross-inhibit and cross-activate each other at some components, e.g. ERK and AKT often phosphorylate the same substrates. Importantly, converging substrate is the MYC signaling, regulated by both pathways.

Due to the importance of the Ras-MAPK pathway in many different cancer types, targeting this pathway is considered as a molecular cancer treatment. RAS inhibition itself did not lead to expected good results, whereas RAF and MEK-inhibitors are already in clinical trials for various tumor types. Selumetinib is a potent but highly selective MEK1/2 inhibitor also called ARRY-142886 or AZD6244. It acts non-competitive with adenosine-triphosphate (ATP) and has no significant inhibitory effect on many other serine/threonine kinases. The inhibitor locks MEK1/2 in an inactive conformation disabling the binding of the substrate and ATP – therefore it blocks the binding to ERK and also the molecular interactions necessary for catalysis. Subsequently, the phosphorylation level of ERK is decreased and leads to increased apoptosis in various cell lines and also inhibits tumor growths in pancreatic xenograft mouse models. Selumetinib is currently in phase I and II studies for a broad range of cancer types, e.g.
colorectal cancer, non-small-cell lung cancer and HCC. In HCC it shows promising anti-tumor activity in combination with sorafenib,\textsuperscript{56} in biliary tract cancer it reached phase two in combination with cisplatin and gemcitabine treatment\textsuperscript{57} and in PDAC a phase II trial showed modest antitumor activity in combination with inhibition of epidermal growth factor EGFR.\textsuperscript{58}

BKM120 is an oral PI3K inhibitor and passed already early phase I clinical trials of advanced cancer patients. BKM120 inactivates a subunit of PI3K, p110α. In solid tumors, the combinatorial treatment of mFOLFOX6 (a common chemotherapeutic backbone) showed increased toxicity with BKM120.\textsuperscript{59} BKM120 has an effect on BTC-cells with wt- or mutated KRAS but not in combination with PIK3CA mutation. To overcome these limitations, the therapy needs to be combined with a MEK-inhibitor.\textsuperscript{60}

4.4.3 \textit{Wnt-signaling network in cancer}

\textit{β-Catenin} is a transcription-cofactor controlling key developmental gene expression programs and is regulated by the wingless and INT-1 (WNT)-signaling. Thus, playing a critical role as a proto-oncogene in embryonic development and adult tissue homeostasis.\textsuperscript{61} At a later time, also the adenomatous polyposis coli (APC) gene was discovered, genetically altered in hereditary cancer.\textsuperscript{62} WNT-signaling is often dysregulated in cancer in one of the two important pathways: the canonical, β-Catenin dependent, and the non-canonical, β-Catenin independent signaling, depending on the activating WNT-ligand and receptor. Both pathways are initiated by the binding of WNT to a receptor on the extracellular membrane, in the canonical pathway that is e.g. frizzled (FZD)-related protein and Low-density lipoprotein receptor (LRP), at which then the proteasome complex of APC, Axin and glycogen synthase kinase 3 (GSK-3) binds. This stops the proteasomal-destruction of β-Catenin and it accumulates in the nucleus, binds transcriptions factors like Lymphoid Enhancer Factor and activates the transcription of target genes involved in cell proliferation, migration, cell cycle regulation and metastasis.\textsuperscript{63}

A positive effector of WNT-signaling is R-Spondin, it binds to leucine-rich repeat containing G-protein-coupled receptors (LGR) 4-6 which inhibits signaling by binding of ZNRF3 or Ring finger protein 43 (RNF43). RNF43 acts as a tumor suppressor as transmembrane E3 ubiquitin ligases, which targets the FZD-receptor in interaction with the protein Disheveled (DSH) for lysosomal degradation.\textsuperscript{40}

The non-canonical pathway is a β-Catenin-independent mechanism where LRP6 will be primed by Cyclin Y/ Cdk14 leading to the activation of the non-canonical cascade in the G2/M cell cycle phase. GSK3β is inhibited and subsequently blocks poly-phosphorylation and poly-ubiquitination of target proteins and their degradation. GSK3β phosphorylates and poly-
ubiquitinates about 20% of proteins of the proteome, including the myelocytomatosis oncogene MYC. Furthermore, the uncanonical pathway affects chromosomal stability, cell division and endolysosomal biogenesis. (reviewed in Zhan et. Al, Oncogene 2017) \[64\]

MYC-oncogenes are a family of three members, genes encoding C-MYC, N-MYC and L-MYC. MYC binds the transcription factor MAX, whereas this heterodimer then recruits a chromatin-modifying complex and in turn activates the transcription via binding the DNA sequence CACGTG (the conserved “E-box”). In addition, C-MYC can bind promoter regions of active genes resulting in transcriptional amplification. \[65\] The function of L-MYC is not well understood and N-MYC expression is tissue-restricted. C-MYC (in this work, MYC refer to MYC) is a proto-oncogene associated with more than 70% of all cancers, but cannot induce tumorigenesis on its own. \[66, 67\] It is found highly expressed in HCC, mostly by genomic amplification \[68\] and is a poor prognosis marker in liver cancer. Activated MYC cooperates with genetic events that synergistically regulate the proliferation-induction and malignant transformation, for example p53, positively correlating that effect to a high proliferative level. \[69\] MYC is a main regulator of the cell cycle arrest and cell growth and plays an essential role during the normal development – so not only drives HCC as an early event but also several different liver diseases. C-MYC regulates a great amount of protein-encoding and non-coding genes involved in a broad range of biological functions like cell proliferation, differentiation, survival and immune surveillance, determined by the cell type, expression level and context.

In PDAC and HCC the WNT-pathway components are often mutated (40-70%) or dysregulated and β-Catenin is localized in the nucleus. \[64, 70\] As described in Section 4.4 and Figure 1, mutation of genes involved in the WNT-signaling pathway are amongst the most frequently mutated genes in HCC, one factor is also the mutation of APC with a frequency of 1.4%. \[71\] HCC arising from normal liver tissue without any precondition (e.g. fibrosis) most frequently present with indications of activation of the WNT/β-Catenin signaling pathway. \[72, 73\]

4.4.4 CDKN2A/CDKN2B locus and its roles in tumor progression

The CDKN2A-locus is also called inhibitor of cyclin-dependent kinase 4/alternative reading frame (INK4A/ARF) locus and is conserved among humans, mice and rats. \[74\] It expresses two tumor suppressor proteins, p16\(^{\text{INK4a}}\) and p14\(^{\text{ARF}}\) called p19\(^{\text{ARF}}\) in mice. The INK4A/ARF locus has an overlapping gene, named ARF and is expressed with a separate promoter but reads two of the same exons in a different frame. Therefore, p14\(^{\text{ARF}}\) does not have amino-acid sequence homology with p16\(^{\text{INK4a}}\) and negatively regulates the p53 destabilizing protein MDM2. p16\(^{\text{INK4a}}\) can inhibit the phosphorylation of Retinoblastoma tumor suppressor protein (RB)
through CDK4/6, resulting in cell cycle arrest via sequestering p53 in the nucleoli and therefore mediating its stabilization.\textsuperscript{75} CDKN2A is often mutated in PDAC, but also downregulated through multiple mechanisms, mostly by genetic deletions and mainly in tumors with high neoplastic cellularity.\textsuperscript{35}

There are mouse models either without exon 2 and 3\textsuperscript{76} or exon 1ß\textsuperscript{77}, showing similar phenotypes, but no mouse model without the whole INK4a/ARF locus. Frequently the whole INK4a/ARF locus is homozygous deleted, including p16\textsuperscript{INK4a}, p14/p19\textsuperscript{ARF}, p15\textsuperscript{INK4b}, MTAP, mir31, DMRTA1 and the α interferon cluster including the interferon ε.

\textbf{4.4.5 Epidermal growth factor receptor ERBB amplifications and mutations in cancer}

Epidermal growth factor receptors called ERBBs are highly conserved and involved in a large number of cancers, either due to dysregulation or mutation of the genes. In mammalians, four different ERBB- (ERBB1 or EGFR, ERBB2 (Her2), ERBB3 and 4) genes are known with a similarity of 40-45% among themselves.\textsuperscript{78} They express receptors which after ligand binding (Epidermal growth factor EGF and Neuregulins or ten other ligands) undergo a conformational change and dimerize with a second ERBB-receptor, subsequently trans-phosphorylating each other and recruiting enzymes and adaptor molecules. Hereby activating the downstream signaling like MAPK and PI3K/AKT pathway, increasing proliferation and inhibition of apoptosis. Erbb2 can dimerize even without ligand binding, which makes it a favorable dimerization partner.\textsuperscript{79}

\textbf{4.5 Genetic manipulation methods}

\textbf{4.5.1 CRISPR/Cas9 System: easy and variable genetic manipulation}

There have been genome-editing technologies available ever since, but as they mostly rely on DNA-protein recognition, the development for new, specific targets is difficult. An effortless genome engineering method brings a breakthrough: the RNA-programmable CRISPR/ Cas9 technology is easy to engineer, scalable and effective, very valuable for tumor biology studies. Clustered regularly interspaced short palindromic repeats/ CRISPR associated protein 9 is a bacterial system that can induce RNA-directed double- or single-strand DNA breaks. In prokaryotes, the Cas9 nuclease is part of the adaptive immune system, which in case of the \textit{Streptococcus pyogenes} SF370 type II CRISPR locus needs four genes: the Cas9 nuclease, the host factor ribonuclease RNase III, a trans-activating noncoding CRISPR RNA (tracrRNA) and a precursor crRNA, containing the nuclease guide sequences interspaced by identical direct
repeats. For introducing double-strand breaks in mammalian chromosomes, nuclear localization signals were attached to ensure the nuclear compartmentalization.\textsuperscript{80}

The CRISPR-repeat-spacer array (crRNA) contains the sequence that complements the target gene sequence, called sgRNA and is introduced with a 3’ end repeat sequence close to a spacer. The complementary sequence is notably located next to a conserved sequence motif on the invading DNA, the 2-5 bp long PAM sequence, which is important for the selection of the targeted DNA sequence.\textsuperscript{81,82} The Cas9 protein assembles with the CRISPR-repeat-spacer array to an effector complex to target the DNA and to destroys the matching sequence. The tracrRNA binds to the repeat sequence at the 3’ site of the crRNA and builds an RNA hybrid structure, guiding the Cas9 to cleave the 20 nucleotides long target sequence with the adjacent PAM sequence. The Cas9-nuclease type II itself has two nuclease domains, the HNH and RuvC domain each cleaving one strand leading to a double-strand break (DSB).\textsuperscript{83,84} This DSB is then repaired either by an error-prone non-homologous end joining (NHEJ), which results in deletion or small random insertion or by high-fidelity homology repair (Homology directed repair, HDR). This causes a heterogeneous population of genetic mutants, also frameshifts or in-frame indels still producing functional proteins are emerging. Additional approaches, besides the single cleavage by Cas9, are large deletions, inversions or even genomic rearrangements using two DSBs or introducing specific mutations as well as genes knock-in mediated by NHEJ. For HDR it is possible to supply a homologous repair template, containing e.g. a specific desired point mutation, gene insertion (or conditional alleles, tags) or gene corrections (reviewed in Jiang et. al, Annu rev Biophys. 2017\textsuperscript{85} and summarized in Figure 2).

Using the CRISPR/Cas9 system to generate deletions in the genome in a vertebrate system was first shown in zebrafish. After transient co-injection of two sgRNAs with Cas9 into zebrafish embryos, a deletion with only low efficiency was reached (1-3\%).\textsuperscript{86} Later on, the CRISPR/Cas9 system has proofed to be a robust system for the production of genomic deletions in mammalian cells. These deletions ranged from 1.3 kb to more than 1 Mb, which then typically have small insertions or deletions at the predicted junctions. Embryonic stem cells (ESCs) showed deletions, inversions and duplications after using two sgRNAs in ESCs up to 1.6 Mb in different clones. Some studies showed a relationship between frequency and size,\textsuperscript{87,88} in others not the size but the locus played a role.\textsuperscript{89,90} An explanation might be the distinct packing of the chromatin at the specific cutting size depending on the epigenetic status. The efficiency of deletion of a region differs from 2.1% to 42%, whereas an inversion (0% - 6.9%) or duplication (0%-28.1%) just barely happens.\textsuperscript{87} Direct injection of the CRISPR/Cas9
components into the zygote lead to detected deletions and inversions from a size up to 1 MB in 22-23% of the cells, rarely also duplications occurs (2 out of 162). These deletions are especially practical for disrupting the function of non-coding regions, gene clusters or regulatory sequences. The disruption of the gene function using the CRISPR/Cas9 system for deletions is more efficient compared to a single cutter because not all single cleavages lead to a frameshift and if, some frameshifts have just the effect of a nonsense-mediated decay or functional isoforms are still produced due to alternative splicing. The CRISPR/Cas9 system dramatically shortens the time to produce changes in the mouse genome but leaves the problem that homozygous mutations on the germline are often lethal on the embryonic level and not tissue specific, therefore the CRISPR/Cas9 system is limited for the study in adult tissue with genetically engineered mouse models (GEMMs). To overcome this, Dow et al. developed a (murine) model using a tetracycline (Tet, doxycycline (Dox)) regulatable system, by which the genetic manipulation can be induced by (feeding) the addition of Dox resulting in the expression of the sgRNA with a Cas9.

Figure 2: CRISPR/Cas9 technology and their results. Cas9 in combination with gRNA binds the target sequence located close to a PAM-sequence and leads to DSB formation. The cell performs repair mechanism (Homology directed repair HDR or non-homologous end joining NHEJ, which can lead to a precise repair (wt), insertion or deletion in frame or not. Picture published on https://www.addgene.org/crispr/guide/.

4.5.2 RNA interference: inducible and reversible knock-down on RNA level

RNA interference (RNAi) is a conserved cellular mechanism for targeted suppression through RNA and describes post-transcriptional gene silencing, either via transfection of small interfering RNA (siRNA) or vector-based stem-loop short hairpin RNA (shRNA). shRNA is 15 to 30 bp long and when bound to the target RNA leads to cleavage by the exoribonuclease Dicer followed by the processing of the RNA-induced silencing complex RISC, the effectors of RNAi. shRNA is expressed by a promoter from RNA Polymerase II, originally expressing miRNA, and can be under the control of a tetracycline response element (TRE).
is the development of effective shRNA-mediated knockdown strains, the variability is high. Therefore, the coupling with a fluorescent reporter is helpful, where using green fluorescent protein (GFP) as a spacer between the TRE and the MIR30 cassette also increases the knockdown efficiency.\(^4\) ShRenilla targets luciferase and serves as a control shRNA, necessary because although the dicer-pathway is highly conserved, the introduction of shRNA into mammalian cells might lead to nonspecific inhibition of translation and cytotoxic responses.\(^5\) RNAi has been studied since many years to make it cost- and time efficient with the nice advantage of inducibility and reversibility overall genetically engineered mouse models.

4.5.3 Cre/lox system

The Cre-recombinase is a 38 kDa big protein recognizing a locus of x-over, p1 (loxP) sites of 34 bp DNA sequence flanking alleles of genes of interests. Cre is a cyclization recombinase of the bacteriophage P1 specific for tyrosine-sites. Mostly, Cre is expressed under the control of a tissue-specific promotor, to excise a floxed loci for the inactivation of the gene only in the tissue of interest. To make the system time-specific, tamoxifen or tetracycline inducible systems are linked to it.\(^6\)

4.6 Pre-clinical Models available in cancer research

Models like the worm \textit{Caenorhabditis elegans}, the fruit fly \textit{Drosophila} and especially the mouse \textit{Mus musculus}, zebrafish \textit{Danio rerio} or the rat \textit{Rattus norvegicus} raised the understanding of the human body and the development of pathologies. However, the translation from these models to human beings in physiologically relevant situations is unsatisfying. Therefore, the need arises for \textit{ex vivo} models to study human diseases appropriately and translationally. Nowadays, due to cost and time efficiency in combination with similarities to the human body, mostly murine models are used.

4.6.1 Two-Dimensional cell lines: basic cellular research

Established two-dimensional (2D) tumor cell lines are easy to maintain and, therefore, often well-characterized. The simple to use system also for studying the molecular mechanism and susceptible to genetic modification leads to fast and reproducible results with low costs. On the contrary, results obtained from 2D cell lines often do not reflect the characteristics and behavior of the tumor as a whole. They differ in terms of genetics, phenotype, and transcriptomic pattern, which can be explained with the adaption of the cell lines to their culture conditions, selecting for mutations and giving advantages to growing in a monolayer. Therefore, they differ sig-
nificantly from their physiological or pathological state before. Still, 2D cell lines are relevant as a proof-of-principle cell line, keeping in mind that further tests in more complex models are required.\textsuperscript{97,98}

The establishment of primary 2D cell lines is difficult, which might also be due to the stringent selection of the culturing conditions and the possible interfering of outgrowth of non-cancer cells. Also, these primary cell lines have some drawbacks – even though they more closely resemble the primary tumor, they still lack cell-cell and cell-matrix interactions. Furthermore, for the establishment of 2D cell lines, a relatively big piece of the tumor is needed, which is why 2D cell lines are only established from tumors of patients who undergo surgical resection.\textsuperscript{99} Currently, there are over 50 established human CCA cell lines\textsuperscript{100} with more upcoming frequently (reviewed in Zach et al., Journal of Stem cell research and transplantation 2015\textsuperscript{101}), and a new one is established for example\textsuperscript{102–106}. For PDAC, way too few cell lines are available to reflect the heterogeneity of the group,\textsuperscript{98} and for human GBC, even fewer cell lines are available,\textsuperscript{107,108} even though the first one was established in 1980\textsuperscript{109}.

4.6.2 The next level of cell culture systems: Organoids

In an organism, cells are in a complex microenvironment embedded in signaling interactions of the extracellular matrix, soluble factor, and mechanical cues. This signaling is defining the development and maintenance of the cellular phenotype and their functions, a possible reason why three-dimensional (3D) culture conditioned cells more closely resemble architectural and functional properties of in vivo tissues compared to 2D cell lines. In 3D cultures, cells contain cell-cell and cell-extracellular matrix (ECM) contacts in a three-dimensional way.\textsuperscript{110} Cells aggregating as 3D structures when growing in non-adherent culture conditions are called spheroids.\textsuperscript{111} Organoids are defined as 3D structures with the potential to self-renew, self-assemble and self-organize into properly differentiated functional cell types of the original organ, recapitulating at least partly the former function of the tissue. Merixxell Huch and colleagues established the culturing of liver and pancreas organoids for mice and humans as well as their genetic manipulation in vitro.\textsuperscript{112} Even a small amount of starting material of GB can form organoids.\textsuperscript{113} They grow out of pluripotent stem cells (embryonic stem cells ESCs), induced pluripotent stem cells (iPSCs) or adult progenitor cells\textsuperscript{114} and can be cultured for long term keeping their genetic stability over time.\textsuperscript{115,116} The 3D cystic structure is growing into a single layer of epithelial cells from a different kind of species, mouse, rat and humans, but needs slightly different culture conditions specified for each. Not only resembling aspects of tissue composition, function and architecture of organs is possible, also individual human diseases ex
vivo can be represented. This is useful in studying diseases on a molecular level as well as to finding personalized treatment options. Therefore, organoids are of great biomedical use, even cell therapy after gene correction is possible.

Defined medium for liver organoids culturing embedded in matrigel needs hepatocyte growth factor HGF, epithelial growth factor EGF, fibroblast growth factor FGF and R-Spondin. For human cells needs the additional regulation of the transforming growth factor-β TGF-β pathway and cyclic adenosine monophosphate cAMP activity. In vitro, depending on the cues given by the extracellular matrix and media, organoids can at least partly differentiate into the different original cell components. Changing into a hepatocyte-differentiation media containing Notch inhibition, removing the R-Spondin and adding dexamethasone and the bone morphogenic protein BMP, the ductal fade is blocked, and cells differentiate into a hepatocyte lineage in humans and mice. Differences between the pancreas and liver organoid culturing is the need of Noggin instead of HGF for the pancreatic organoids. GB and liver organoids are cultured following the same protocol.

Gene expression profiles of liver organoids showed next to progenitor markers like LGR5, transcription factor Sex determining region Y-box 9 (Sox9), cluster of differentiation 44 (Cd44) and Cd133 a bipotential phenotype. The profiles are expressing a mixture of ductal-markers like Cytokeratin 7 (CK7), CK19, and hepatocyte markers like Transthyretin (Ttr) and hepatocyte nuclear factor-4α (Hnf4α), but only on a low-level marker for mature hepatocytes. GB epithelial cells growing as organoids have a similar bipotential character, as well as pancreatic organoids having an overlapping transcriptome profile with epithelial bipotent progenitor cells. GB and pancreas organoids both can be differentiated in vitro into hepatocytes and be transplanted into mice, where they engraft. Another marker expressed by GB and liver organoids in culture is the epithelial cell adhesion molecule (EpCAM), which has a proliferative effect and also regulates the actomyosin network as well as cell-cell contact. The adhesion protein is expressed by epithelial precursor cells, undifferentiated stem cells and immature liver cells but gets lost after differentiation to hepatocytes.2

PDAC, GBC, PLC – either HCC, CCA and mixed subtypes – can be established as organoids and are then called tumoroids. Similar to physiological tissue, they resemble and keep the gene expression with their genetic alterations over time. As well, when reinjected into immunocompetent mice, they closely reflect the histological architecture and recapitulate the metastatic potential. When there is the possibility to establish healthy liver organoids side by side, genetic changes playing a role in the tumor can be identified, and effective personalized
therapy with small side effects can be found. The heterogeneity of the tumoroids and their different sensitivity shows the importance of these studies.2

Liver cancer organoids could be established only by a small amount of material acquired by a needle biopsy, and especially GB and GBC organoids need only small amounts as starting material.2,113,121,123 Still, the low efficiency of establishing primary human organoid cell lines is problematic, which is lower in well-differentiated tumors. One explanation could be that the well-differentiated tumors have only a few proliferating cells. This is the case in GBC and CCA, which contain a high number of stroma cells, interfering in the culturing of the proliferating cells. Saito et al. showed that non-cancerous organoids are more robustly proliferating than tumoroids. Hence, some "contaminating" non-cancerous cells in organoid cultures overgrow the cancerous culture.121

4.6.3 Transplantation of cell lines into mice

Animal models are a very powerful tool to study diseases in a whole organism and to investigate the interaction between cancer cells and the non-neoplastic organismal environment, for example, stromal cells, angiogenesis and immune system. Transplanting cell lines into a living organism is an easier and faster method than engineering mouse models. Injected cell lines from one individual to another of the same species, is called an allograft, which can be syngeneic when the individuals are genetically identical or at least closely related. If cells are injected into a different species, the model is called xenograft, which is often used as a patient-derived xenograft, where patient-derived material is injected into immunocompromised mice. The injection can be orthotopic, at the same organ as the cell type belongs to, or ectopic, whereas here for easier accessibility mostly subcutaneous injections are used.124

Cells lines injected into mice positively select for the most aggressive clone which, in turn, leads to an only low recruitment of a desmoplastic stroma – they are more likely to grow in a homogenous mass.125 Patient-derived xenografts (PDX) should overcome these disadvantages with transplantation of a large piece of a human tumor into mice, keeping a part of the microenvironment and morphological characteristics of the original tumor as well as the metastatic potential.126,127 These lead to a response to chemotherapeutics closely mirroring the human response,128 but out of all resectable tumors only very few and mainly very aggressive tumors engraft in mice after PDX transplantation. Thus, this is predicting negative disease progress for the patient.129,130 Instead of injecting 2D tumor cell lines, tumoroids (3D cultured tumor cell lines) from PDAC, GBC and CCA develop tumors resembling histopathological the glandular and tubular structures of the adenocarcinomas.
Using the CRISPR/Cas9 system made it easier to produce syngeneic allograft mouse models. Cancerous cells or solid tumors derived from the same genetic strain can be created and reinjected – a mouse model in which the effect of the immune system on the tumor can be studied. Therefore, the only disadvantage is the rodent source of the tumor cells and organism, which does not completely recapitulate the human disease.\textsuperscript{131,132} Freshly isolated fetal liver cells can be genetically modified in culture, transplanted into recipient mice and depending on the genetic context, lead to either HCC or CCA. Murine fetal liver cells harboring the Cre-recombinase Alb-Cre and p53\textsuperscript{LslR172H/lox} possess a mutated p53 expressed in liver progenitor cells and adult hepatocytes from one chromosome whereas the gene is deleted on the other chromosome, resembling a typical situation in patients. In combination with an overexpressed mutated KRAS\textsuperscript{G12D} injected orthotopically into mice, this results in CCA-resembling histology. Mutated p53-fetal liver cells in combination with overexpression of c-MYC on the contrary result in a different histology without the CCA-characteristical stromal reactions and more likely resemble HCC.\textsuperscript{133}

4.6.4 Transgenic mouse models for preclinical cancer studies

Even though 3D cell lines can display cell-cell contact and co-culturing with different cell types is possible, it does not resemble the whole complex environment of an organism. Problematic on modeling PC is that upcoming data on human cancer shows the uniqueness of every human tumor, making the use of genetically engineered mouse models (GEMMs) or cell lines less meaningful.\textsuperscript{134} Still GEMMs are valuable for studying tumor biology in the case of tumor development, maintenance, metastasis and therapy effect with intact stroma and immune cells, with the need of keeping in mind that it is still a murine model, with distinctions to humans (e.g., different telomere properties, different chromosomal location).\textsuperscript{125} In addition, the next-generation GEMMs are developed, in which a recombinase-driven system is not only introduced in cancer origin cells, but also in the whole body or specific cell lines of the stroma, coupled with inducible promotors for studying the effect of gain and loss at different time points.\textsuperscript{135} Even though the knowledge of the cancer genome constantly increases with concurrently the rationale for personalized therapy, there is still the need for \textit{in vivo} models to understand how cancer driver act or their dependencies impact the tumor and treatment response. GEMMs resemble human cancers with the different stages of development and pre-neoplastic lesions, making studies in tumor development and their early detection possible. Still, they are costly and time-consuming to produce and, therefore, not beneficial for representing the heterogeneity of PLC, BTC or PC.
4.6.4.1 Genetically engineered mouse models – GEMMs

For CCA, only a few different mouse models are available and many displaying a mixed CCA/HCC histology.\textsuperscript{136-138} Having a partly CCA histology but mainly HCC was found in mice carrying liver-specific p53 and Ink4a/Arf, loss or inactivation of NF2, Sav1 or Mst1/Mst2 tumor suppressor genes.\textsuperscript{139} The cell-specific loss for murine liver cancer models is mostly controlled by the hepatocyte-specific Albumin expression of Cre-recombinase.\textsuperscript{139} In addition, with a defined genetic context, these alterations can lead to CCA development: Smad4 and Pten deletion lead to CCA development in four to five months, mice with Kras activation and Pten loss have a fast tumor development of seven weeks, activated Kras with IDH-mutations show CCAs with a mean survival of 47.3 weeks\textsuperscript{139} and carrying activated Kras and Tp53 loss show latencies of 9-19 weeks. Cholangiocyte-specific Kras activation and TP53 loss also lead to CCA, but with a significantly prolonged latency of 30 weeks. Also, the dysregulation of the Notch-signaling, for example, overexpression of Notch1 intracellular domain NICD in combination with Tp53 deletion, presents CCA in mice with high penetrance, but long latency of eight to nine months.\textsuperscript{100} Formation of CCA can also develop from hepatocytes when Notch signaling is activated in combination with AKT overexpression.\textsuperscript{140} Expressing an activated form of Notch1 in combination with mutated Kras also represents a useful preclinical model for CCA development.\textsuperscript{141}

Spontaneous gallbladder cancer in mice is rare, but there is some chemically induced neoplastic lesion in the murine gallbladder with still low incidence. A mouse model overexpressing a rat ERBB-2 under the control of the bovine keratin five promotor developing GBC is described as a first efficient mouse model for GBC.\textsuperscript{42}

Since KRAS is activated in almost all PDACs, most GEMMs also rely on that. In combination with altered tumor suppressor genes, mice develop PDACs. Mutations in Tp53 or loss of Smad4, Brca2 or Cdkn2a accelerate the PDAC development and lead to tumors with different histologies.\textsuperscript{142} Mostly used is the KPC model, in addition to the mutation in KRAS (mostly Kras$^{G12D}$, activated through the excision of a stop codon in front of its endogenous promotor) loss or mutational deactivation of the floxed Tp53 that is introduced in a pancreas-specific way: The recombinase Cre is expressed under the control of the Pdx1 or Ptf1a promoter, which is only expressed in cells of the pancreas from the embryonic status on.\textsuperscript{49,143} Mutated Kras alone leads to ductal lesions, comparable with human PanINs. In combination with depleted p53, mice develop PDACs with a substantial desmoplastic stromal reaction, immune cell infiltration and hypovascularity.\textsuperscript{49} Using the KPC model (LSL-Kras$^{G12D}$; lsl-p53$^{R172H}$; Pdx1-Cre) to study...
the effect of an additional gene needs time-consuming intercrossing ending in multi-allelic mice harboring four engineered alleles.

4.6.5 *Embryonic stem cell-based – GEMMs: technical approach*

Next to the breeding strategies to produce new GEMMs, stem cell technologies have enabled the production of mouse strains harboring multiple alleles without the need of any intercrossing. Derivating embryonic stem cells (ESCs) from mice already harboring conditional disease-associated alleles can be genetically modified *in vitro* and, in turn, used to create a cohort of experimental mice by blastocyst injection or tetraploid complementation. Efficient transgenesis approaches are invented, for example, a recombinase-mediated cassette exchange strategy (RMCE). The RMCE-strategy allows rapid introduction of a defined genetic locus, where the locus can contain genetic elements expressing shRNA, overexpression of oncogenes or a CRISPR/Cas9 implemented interruption of gene expression coupled with a fluorescent reporter under a tet-responsiveness expression cassette.

For the PDAC-model described in this work, previously described KC-RIK ESCs to produce GEMMs are used. Shortly, the ESCs include a cre-recombinase under the control of Ptf1a promoter and a heterozygous Kras\(^{lslG12D}\) allele (KC). Further, the ESCs harbor an allele co-expressing mKate with a reverse tetracycline transactivator (TRE) upon cre-activation. Here, Cas9 expression is linked to GFP under a tetracycline regulatable promoter, whereas the sgRNA is constantly expressed. The tet-transactivator, in combination with doxycycline treatment, lead to the expression of Cas9 linked to GFP, genetically manipulating genes dependent on the constantly expressed sgRNA target sequence.

4.6.6 *Electroporation: transfection of adult liver or pancreas in vivo*

One method developed by the lab of Florian Kühnel at Hannover Medical School is the electroporation-based transfection method into the liver or pancreas *in vivo*. This method is useful in order to generate genetically highly flexible transgenic mouse models in a timely and economical manner. After injecting a DNA plasmid mixture into the organ, an electric impulse transfers the DNA into the cells. Activating the two most frequent genetic alterations in PDAC in the mouse model Kras\(^{lslG12D\text{wt}}\); p53\(^{fl/fl}\) only by electroporating a Cre-recombinase leads to locally restricted tumors with a median survival of 84 days. In the pancreas, they showed in early stages PanINs and later on PDACs moderately differentiated expressing CK19 and desmoplastic stroma surrounding the tumor cells, resembling the human disease PDAC. The liver electroporation approach is published in combination with a CRISPR/Cas9 based sgRNA
screen, where investigations of metastasis in the mouse model, tracking metastatic tumors comparing the indel-/deletion formation with the primary tumor is possible. At that time, the paper was the first one describing somatic engineering of inter-chromosomal translocations in a higher organism.\textsuperscript{150}

4.6.7 \textit{High dynamic tail vein injection HTVI}

Hydrodynamic tail vein injection (HTVI) is the injection of high amounts of plasmid-DNA in a fast and high-pressure injection into the tail vein, leading to the transfection of 2-10\% of all hepatocytes. HCC is the favorable tumor subtype to study, maybe because mainly hepatocytes are affected. But also CCA can be studied – e.g., dysregulation of the Notch pathway in combination with other common genetic events, like a disruption of the KRAS driven pathway, lead to CCA even though hepatocytes are targeted via HTVI.\textsuperscript{140,141,151,152} CRISPR/Cas9 system has proven to work either for the characterization of tumors with the disruption of \textit{p53} and \textit{Pten} is working,\textsuperscript{153} as well as the possibility for genetic screening with up to ten sgRNAs.\textsuperscript{154} Advantages are that only a part of the cells is targeted with tumorigenic genetic alterations so that they can still interact with a normal and healthy microenvironment.\textsuperscript{100}
5 Manuscript I: Murine Liver Organoids as a Genetically Flexible System to Study Liver Cancer In Vivo and In Vitro, Hepatology Communications, 2019

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Murine Liver Organoids as a Genetically Flexible System to Study Liver Cancer In Vivo and In Vitro

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The rising incidence of cholangiocarcinoma (CCA) coupled with a low 5-year survival rate that remains below 10% delineates the urgent need for more effective treatment strategies. Although several recent studies provided detailed information on the genetic landscape of this fatal malignancy, versatile model systems to functionally dissect the immediate clinical relevance of the identified genetic alterations are still missing. To enhance our understanding of CCA pathobiology and facilitate rapid functional annotation of putative CCA driver and tumor maintenance genes, we developed a tractable murine CCA model by combining the cyclin D1 recombination (Cre) system, RNA interference, and clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9 (CRISPR/Cas9) technology with liver organoids, followed by subsequent transplantation into immunocompetent, syngeneic mice. Histologically, resulting tumors displayed cytokeratin 19–positive ductal structures surrounded by a desmoplastic stroma—hallmark features of human CCAs. Despite their initial biliary phenotype in vitro, organoids retained the plasticity to induce a broader differentiation spectrum of primary liver cancers following transplantation into recipient mice, depending on their genetic context. Thus, the organoid system combines the advantage of using nontransformed, premalignant cells to recapitulate liver tumorigenesis as a multistep process, with the advantage of a reproducible and expandable cell culture system that abrogates the need for recurrent isolations of primary cells.

Conclusion: Genetically modified liver organoids are able to transform into histologically accurate CCAs. Depending on the oncogenic context, they are also able to give rise to liver cancers that show features of hepatocellular carcinoma. The model can be used to functionally explore candidate cancer genes of primary liver cancers in immunocompetent animals and evaluate novel treatment regimens. (Hepatology Communications 2019;3:423–436).

Three-dimensional (3D) organoid cultures have been derived successfully from a variety of organ systems and cell types, such as intestinal cells, cerebral tissues, and, more recently, liver cells.10 These culture systems exhibit commitment to the respective tissue of origin and faithfully retain their genetic profiles, even after long-term expansion.10 Although the exact localization of the liver

Abbreviations: 3D, 3-dimensional; 2D, 2-dimensional; CCA, cholangiocarcinoma; CK19, cytokeratin 19; CRISPR, clustered regularly interspaced short palindromic repeats; HCC, hepatocellular carcinoma; Kras, Kirsten rat sarcoma oncogene; lollis, liver-stage-bee; Mips, myeloid-specific oncogene; p66/62 MAPK, p66/62 mitogen-activated protein kinase; Pten, phosphatase and tensin homology; sRNA, single-guide RNA; shRNA, short hairpin RNA; sRNA, single-guide RNA; WT, wild type.

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organoid-initiating cell remains elusive, the organoids cultured under conditions described by Huch et al. likely originate from epithelial cell adhesion molecule (EpCAM) bile duct epithelial cells and not from hepatocytes, the most abundant cell type in the liver. 2,3 Consequently, these cells readily express bilary markers in vitro. Seminal work recently demonstrated that, apart from normal liver tissue, organoids can be established from human liver cancer specimens, and organoids derived from hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), or mixed subtypes are able to maintain the genetic and histological hallmarks of the initial tumor specimen in vitro. 4,5

CCA is a dismal malignancy that is highly refractory to current chemotherapeutic regimens and targeted therapies. At the time of diagnosis, most patients present with advanced disease not amenable to surgical resection. 5 Histologically, CCA commonly exhibits a ductal growth pattern with an abundant fibrous stroma.

Despite an increasing wealth of sequencing data from human tumor specimens and the identification of recurrent genetic alterations, as well as key prognostic transcriptome signatures, detailed understanding of actionable molecular alterations remains elusive. 6,9 Here, we describe how primary murine liver organoids can be used to study CCA in vivo and in vitro and aid in the translational annotation of the CCA mutagenome.

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Materials and Methods

Additional information, including antibodies, sample preparations, introduction of short hairpin RNAs (shRNAs) and single-guide RNAs (sgRNAs) into organoids, bioinformatic analyses, and statistical procedures, is provided in the Supporting Information.

ANIMAL EXPERIMENTS

All experiments involving mice were performed according to animal protocols approved by local authorities (the Lower Saxony State Office for Consumer Protection and Food Safety). Animals were maintained under standard housing conditions with a 12-hour day-night cycle and access to food and water ad libitum. All interventions were performed during the day cycle. Recipient mice (C57BL/6) and NSG [CD1-Prkdcre;Idd12gTg(10166)Szm] 5-8 weeks old were obtained from the local animal facility (Hannover Medical School, Germany). Kirsten rat sarcoma virus oncoprotein KrasG12D; p53+/− mice were a gift from Dietrich Saur (Munich, Germany), and KrasG12D; 53+/− mice were a gift from Florian Kühnel (Hanover, Germany). C57BL/6 mice were used as recipients for organoids derived from C57BL/6 or syngeneic KrasG12D; p53+/− mice. Organoids derived from KrasG12D; p53+/− animals (mixed background) were implanted into NSG mice.
ISOLATION OF MURINE LIVER ORGANOIDs

Murine organoids were isolated from adult C57BL/6j mice, 
Kras<sup>G12D</sup>; p53<sup>fl/fl</sup> mice, or Kras<sup>G12D; p53<sup>fl/fl</sup></sup> mice according to published protocols. Briefly, murine livers were minced and enzymatically digested in Earle's Balanced Salt Solution (EBSS; Thermo Fisher, Waltham, MA) containing 2.5 mg/mL Collagenase Type IV (Sigma-Aldrich, St. Louis, MO) and 0.1 mg/mL Dnase I (Sigma-Aldrich) for 20 to 40 minutes with repeated pipetting and washed through a 70-μm cell strainer. After addition of washes, cells were spun at 300g, resuspended in 100% Growth Factor Reduced Matrigel (Corning, NY), and plated (two 50-μL droplets per 24-well). After solidification, Matrigel droplets were overlaid with 500-μL murine liver organoid media according to published protocols. Alternatively, residual fragments retained in the cell strainer after collagenase digest were plated in Matrigel. For passaging, organoids were mechanically disrupted by repeated pipetting using a P200 pipette tip, followed by a 5-minute to 8-minute enzymatic digestion in TrypLE Express solution (Thermo Fisher).

TUMOR CELL ISOLATION

Tumors were minced and enzymatically digested in a shaking incubator with Collagenase IV 1 mg/mL (Sigma-Aldrich) in EBSS (Thermo Fisher) for 30 minutes at 37°C. Cells were washed, spun at 300g, and resuspended either in Matrigel and overlaid with organoid expansion medium for organoid culture or resuspended and plated on tissue culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin for 2-dimensional (2D) culture.

CELL VIABILITY ASSAY

Kras<sup>G12D; p53<sup>fl/fl</sup></sup> and Kras<sup>G12D; p53<sup>fl/fl</sup></sup> organoids were seeded in 100-μL 10% Matrigel per 96-well. Two, 24, and 48 hours after seeding, the organoids were lysed and CellTiter-Glo 3D Luminescent Cell Viability Assay (Promega, Madison, WI) was performed according to the manufacturer's protocol.

For inhibitor treatment, Kras<sup>G12D; p53<sup>fl/fl</sup></sup>; LMP<sub>sh</sub>Renilla 713 tumor cell lines established as 2D or 3D cultures from primary, organoid-derived tumors were plated at 1,000 cells (2D cell line) and 10,000 cells (3D cell line) per 96-well and treated with selumetinib (3.36 μM; MedChem Express, Monmouth, NJ), NVP-BKM120 (0.25 μM; MedChem Express), or a combination of both inhibitors for 24 hours or 48 hours. At the indicated time points, cells were lysed using CellTiter-Glo Luminescent Cell Viability Assay or CellTiter-Glo 3D Luminescent Cell Viability Assay, and luminescence was acquired on a Glomax Multi Detection System (Promega, Madison, WI).

FLOW CYTOMETRY

Single cell suspensions from murine livers or organoids were prepared and incubated with primary antibody (1:100 dilution) for 30 minutes at 4°C (Allophycocyanin-EpCAM; Thermo Fisher, Cat. #17-5791-80). Flow cytometry was performed on a FacsCanto (BD Biosciences, San Jose, CA).

Results

EXPRESSION OF ONCOGENIC KRAS IN COMBINATION WITH P53 LOSS IN TRANSPLANTED LIVER ORGANOIDs GIVES RISE TO CCA IN RECIPIENT MICE

We previously developed a murine model for CCA based on the transplantation of fetal liver cells. Because fetal liver cells cannot be propagated long term in vitro, the model required the continuous maintenance of multi-allelic colonies, timed matings, and isolation of primary cells. We aimed to evaluate the use of liver organoids to create a CCA model that eliminates the need for repetitive fetal liver cell isolations while maintaining the advantage of using nontransformed, premalignant cells to recapitulate tumorigenesis as a multistep process.

The Kras oncogene and the tumor suppressor Tp53 are among the most frequently mutated genes in CCA. Hence, we isolated organoids from adult Kras<sup>G12D; p53<sup>fl/fl</sup></sup> mice and cultured them according to published protocols. As anticipated, we could detect expression of cytokeratin 19 (CK19), cytokeratin 7 (CK7), sex determining region Y (SRY)-box 9, and EpCAM, in line with a biliary phenotype of liver organoids in vitro (Fig. 1 A-B; Supporting Fig. S1).
Interestingly, when we slightly modified the isolation procedure by plating the fragments that remained after collagenase digestion instead of plating liver cell suspensions, organoids budded abundantly from the fragments, leading to an accelerated establishment of stable organoid cultures (Supporting Fig. S2).

To activate the latent Kras allele and excise the p53 allele, we transduced the organoids with a retroviral vector containing a tamoxifen regulatable Cre-recombinase and a neomycin resistance cassette (Fig. 1D), and confirmed excision of the respective lox sites by polymerase chain reaction (PCR).

**FIG. 1.** Murine liver organoids express markers of biliary differentiation. (A) Immunofluorescence staining of organoids reveals expression of biliary markers such as CK7, CK19, and SOX9. Scale bars correspond to 50 μm. (B) The adult murine liver contains only a minor fraction of EpCAM-positive cells as determined by flow cytometry (left histogram), whereas nearly all liver-derived organoids are EpCAM-positive (right histogram). (C) Activation of a latent Kras\(^{G12D}\) and loss of p53 lead to increased proliferation as assessed by cell viability assay. (D) Schematic of the retroviral vector pSIN-PGK-CreERT-IRES-Neo\(^r\) that was used to activate the latent Kras allele and delete p53 following tamoxifen treatment. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; RLU, relative light units; and SOX9, sex determining region Y (SRY) box 9.
(Supporting Fig S3). Following activation of the latent alleles, proliferation of the organoids increased without any overt morphological alterations (Fig 1C and Supporting Fig S1B).

Next, we aimed to determine whether transplantation of the genetically modified organoids leads to tumor development in recipient mice (Fig 2A). Following subcutaneous (s.c) injection, tumors developed with 100% penetrance and were harvested after 8 weeks. Consistent with activation of mutant Kras and loss of p53, Cre-recombined organoids before transplantation as well as organoids derived from resulting tumors (tumoroids) exhibited increased phosphorylated p44/42 mitogen-activated protein kinase (p-p44/42 MAPK) levels and loss of p21 (cyclin-dependent kinase inhibitor 1A) expression (Fig 2C). Histological examination uniformly revealed gland-forming adenocarcinomas with CK19-positive tumor cells surrounded by an abundant stromal reaction, compatible with moderately differentiated CCA (Fig 2B).

ORGANOID ALTER THEIR GENETIC AND MOLECULAR PROFILES DURING NEOPLASTIC TRANSFORMATION

To delineate whether liver organoids acquire additional genetic alterations following genetic modification during in vitro culture and in vivo tumorigenesis, we performed genomic copy number analysis of Kras<sup>G12D/somatostatin</sup> p53<sup>fl/fl</sup> organoids before injection into recipient mice and of organoids derived from the resulting tumor in comparison to the parental wild type (WT) organoids. In vitro activation of mutant Kras and loss of p53 led to multiple genomic deletions and amplifications in pre-injection organoids and even more so after in vivo tumorigenesis in the isolated tumoroids (Fig 2E). Several of these alterations correspond to regions with oncogenic potential altered in human CCA patient samples (Supporting Table S1).

Next, we performed whole transcriptome analysis to assess transcriptome profiles of WT organoids and Cre-activated pre-injection organoids as well as tumoroids. Unsupervised cluster analyses revealed that WT organoids and Cre-activated pre-injection organoids shared similar expression profiles, whereas tumoroids exhibited a markedly distinct expression profile (Fig 2D). Gene set enrichment analysis (GSEA) demonstrated that during the progression from wild type to pre-injection cells, gene sets associated with cell cycle regulation, E2F and myelocytomatosis oncogene (MYC) target genes were activated, in line with increased proliferation of the genetically modified organoids observed in vitro (Fig 1C). Tumoroids isolated from murine CCAs exhibited activation of several gene sets associated with inflammation and paracrine signaling when compared with pre-injection organoids, which might be of importance during tumor progression (Supporting Fig S4).

LIVER ORGANOIDS FOR PRECLINICAL DRUG TESTING IN VIVO AND IN VITRO

Therapeutic approaches are limited in patients with CCA, and first-line chemotherapy includes gemcitabine-based treatment protocols. Therefore, we tested the effect of gemcitabine on tumors arising in our murine CCA model. Similar to human patients, gemcitabine treatment led to a moderate survival benefit (median overall survival gemcitabine versus vehicle: 42 days versus 32 days; Supporting Fig S3), but not to complete tumor regression, suggesting that the model is suitable for preclinical drug testing in an immunocompetent in vivo situation.

Apart from more complex in vivo model systems, tumor cell lines are an indispensable tool to study the molecular pharmacology and explore the efficacy of anticancer drugs in a preclinical setting.

To assess the scalability of the system for drug testing, we first tested whether the system is suitable to generate cancer-derived cell lines that are serially transplantable, while maintaining the histological features of the primary malignancy. Tumors derived from s.c. injected Kras<sup>G12D/somatostatin</sup> p53<sup>fl/fl</sup> organoids were collagenase-digested, and cells were cultured and passaged either under standard 2D cell culture conditions in serum-containing media or kept as 3D tumoroid cultures over 14 days (Fig 3A). Both 2D and 3D cell lines gave rise to predominantly moderately differentiated CCAs with similar relative stromal content (CK19-negative area) following s.c. transplantation (Fig 3A-C). Thus, the organoid model can be used to generate well-expandable, genetically defined 2D and 3D tumor cell lines that maintain the histology of the primary tumor following retransplantation. Next,
we interrogated whether in vitro susceptibility toward targeted therapies depends on the culture condition. Combinatorial treatment with mitogen-activated protein kinase kinase (MAPKK) and phosphatidylinositol 3-kinase (PI3K) inhibitors has been suggested for Kras mutant cancers\(^{(15,16)}\) and CCA.\(^{(17)}\) Genetically engineered tumor-derived cell lines established in parallel as 2D versus 3D cultures from the same Kras
FIG. 2. Genetically modified liver organoids give rise to CCA in vivo. (A) Technical outline. Murine organoids lead to tumor formation in recipient mice following Cre-mediated activation of mutant Kras and loss of the tumor suppressor p53. (B) Tumors exhibit glandular differentiation and stromal desmoplasia, resembling human CCA. CK7 and CK19 expression in tumors confirm ductal differentiation. Acanth blue staining indicates elevated production of mucins. Cluster of differentiation 31 stains blood vessels, and sinus red/fast green confirms the presence of collagen fibers. Scale bars correspond to 200 μm. (C) Immunohistochemistry shows p-p44/42 MAPK up-regulation in mutant Kras expressing organoids and p21 down-regulation following loss of p53. (Genotypes of organoids before Cre activation and after Cre activation are indicated.) Asterisks mark tumor-derived cell line. (D) Expression profiles of KRAS/12G12D; p53+/− (wild-type) organoids, KRAS/12G12D; p53−/− (pre-injection) organoids, and organoids derived from tumors of transplanted KRAS/12G12D; p53−/− organoids (tumors). Unsupervised cluster analyses were performed based on differentially expressed genes using Euclidean distance. (E) Array comparative genomic hybridization analysis reveals acquisition of copy number alterations after genetic modification of organoids and during in vivo tumorigenesis. Red, deletion; blue, amplification. Lane 1: KRAS/12G12D; p53−/− pre-injection versus KRAS/12G12D; p53−/− organoids (WT). Lane 2: KRAS/12G12D; p53−/− tumours versus KRAS/12G12D; p53+/− organoids (WT). Lane 3: KRAS/12G12D; p53−/− tumours versus KRAS/12G12D; p53−/− organoids (pre-injection). Abbreviations: CCGH, array comparative genomic hybridization; CD133, cluster of differentiation 33; Chr, chromosome; CNAs, copy number alterations; HeE, hematoxylin and eosin; lii, low-stop-low; p21, cyclin-dependent kinase inhibitor 1A; and p-p44/42 MAPK, phosphorylated p44/42 mitogen-activated protein kinase.

...mutant murine CCA were treated with a MAPKK inhibitor (selumetinib) and a PI3K inhibitor (BKM-120), alone and in combination. Independent of the culture conditions, both 2D and 3D cultures exhibited a comparable response to targeted therapy in vitro, as assessed by luminescence assay (Fig. 3D), indicating that, although organoids can be conveniently used to engineer genetically defined primary tumors, 2D cell lines derived from these tumors are a viable tool for in vitro drug studies. Cell cycle analysis revealed that 2D and 3D cell lines with the respective drugs caused an accumulation of cells in the gap 0 phase/gap 1 phase (G0/G1 phase) and a concomitant S-phase reduction (Fig. 3D, lower panel). Cell lines derived from non-Kras mutant tumors did not exhibit comparable growth inhibition (Supporting Fig. S8C).

LIVER ORGANOIDS CAN BE USED TO PROBE CANDIDATE CANCER GENES IN VIVO

The advent of next-generation sequencing technologies led to the identification of the mutational landscape of human CCA. However, the relevance of individual genetic alterations for initiation and maintenance of the tumors is still unknown. To probe whether our model is suitable for the functional characterization of candidate tumor suppressor genes, we retrovirally introduced an shRNA directed against the tumor suppressor phosphatase and tensin homolog (shPten) or a nontargeting control (shRenilla) into Kras/12G12D; p53−/− recombined organoids. Pten knockdown activated thymoma viral proto-oncogene (Akt) signaling (Fig. 4C) and markedly accelerated tumor growth compared with control–transduced cells (Fig. 4A). Histologically, tumors from both groups were indistinguishable and not altered by loss of Pten (Fig. 4B,D). This experiment highlights the ease with which candidate cancer genes can be probed by RNA interference–mediated knockdown. Of note, a comparable transgenic approach would have required breeding six different transgenic alleles (e.g., Albumin–Cre; Kras/12G12D; p53−/−; Pten−/−).

The shRNAs were expressed from a retroviral backbone-LMP co-encoding for a fluorescent reporter gene (green fluorescent protein [GFP]) that, in addition to testing candidate cancer drivers, allows tracing of cells derived from the transplanted organoids in the recipient animal. The stroma cells were GFP-negative, indicating that stroma cells were exclusively derived from the recipient host and not from epithelial-to-mesenchymal transition of tumor cells (Fig. 4D). Stromal content did not differ significantly in tumors with and without loss of Pten (Supporting Fig. S6).

To address whether the prominent stromal reaction is a direct consequence of injecting the Kras mutant organoids into the subcutaneous microenvironment or whether recruitment of stromal cells is rather an intrinsic feature of the transplanted organoids, we orthotopically injected organoids into the liver. Despite the profoundly different injection sites, tumors developing within the liver were histologically comparable to tumors that formed from sq-injected organoids. In both settings, the organoid–derived, Kras mutant tumor cells were able to recruit stromal cells and induce a desmoplastic reaction (Supporting Fig. S7A,B).

In contrast to RNA interference, CRISPR/Cas9 technology facilitates genome editing and allows for...
FIG. 3. Organoid-derived tumors are serially transplantable as 2D or 3D cell lines. (A) Genetically defined tumor cell lines established as 2D or 3D (tumoroid) cultures from primary, organoid-derived xenografts. (B) Differentiation grading of tumors derived from murine-rejected 2D cell lines and tumors. (C) Stromal content of 2D and 3D cell line–derived tumors does not differ significantly. The CK19-negative area was used as a surrogate for stromal content. (D) The efficiency of targeted therapy does not depend on the culture conditions. Both 2D and 3D cultured tumor cell lines show a comparable response to targeted therapies in vitro. Cell-cycle fluorescence-activated cell sorting reveals the accumulation of cells in the G0/G1 phase and a concomitant S-phase reduction following drug treatment in the 2D cell line. Abbreviations: FACS, fluorescence-activated cell sorting; G1, grade 1; G2, grade 2; G3, grade 3; and NS, not significant.

complete loss of function or the generation of genomic deletions. To introduce genetic perturbations into live organoids using CRISPR/Cas9, we transfected a plasmid co-encoded for a Cre-recombinase, Cas9 protein, and sgRNAs directed against the tumor suppressors Pten and p53 into Kras\textsuperscript{G12D/+} organoids (sgP53/ sgPten-CG\textsuperscript{118}) (Fig. 4E, top panel). Cre-mediated recombination was confirmed by PCR and efficient target cleavage by T7 endonuclease assays (Fig. 4E, left panel). Similar to their shRNA-transgenic counterparts, these organoids gave rise to CK19-positive, moderately differentiated murine CCAs (Fig. 4F). Isolated tumors showed complete loss of p53 and Pten expression (Fig. 4E, right panel). In summary, liver organoids can be genetically modified with either RNA interference or CRISPR/Cas9 technology to interrogate gene function in vivo, and fluorescent marker proteins facilitate the tracing of transplanted cells in the recipient animal.

ORGANIODS EXPRESSING A BILIARY MARKER PHENOTYPE ARE ABLE TO GIVE RISE TO TUMORS RESEMBLING HCC FOLLOWING MALIGNANT TRANSFORMATION

Next, we assessed whether the intrinsic biliary marker constellation expressed by the organoids in \textit{vitro} translates into the development of tumors of biliary differentiation or whether the organoids retain the plasticity to give rise to a broader differentiation spectrum. We transduced organoids derived from WT C57Bl/6 mice with retroviruses encoding for murine myelocytomatosis oncogene (Myc) and co-expressing a red fluorescent reporter (mCherry), an shRNA directed against p53 coupled to a GFP reporter, and transfected an sgRNA targeting \textit{Aneur} (adenomatous polyposis coli) (Fig. 5A, and Supporting Fig. S8A). Intriguingly, substituting Kras with Myc as an oncogenic driver resulted in tumors of a different and distinct differentiation: Unlike the duct-forming, stroma-rich adeno- carcinomas that arise in the context of mutant Kras, Myc-overexpressing tumors display a compact, solid growth pattern of tumor cell nests lacking prominent desmoplasia. The tumor cells stained negative for CK19 and exhibited more prominent nuclei, overall more resembling HCC than CCA (Fig. 5C).

Comparative analyses of the CCA tumors and the HCC-like tumors by RNA sequencing identified 4,723 differentially expressed genes. Unsupervised clustering confirmed the distinct gene expression profile of Myc overexpressing and Kras mutant tumors (Fig. 5D). Consistently, GESEA (Fig. 5E) confirmed that Kras-mutated tumors were predominantly driven by activation of Kras-dependent gene sets, whereas HCC-like tumors showed an activation of MYC target genes, overall matching the differences in histology and thus validating the approach presented here. Similar to what we observed in CCA organoids (Fig. 2D), Myc-overexpressing pre-injection organoids and WT C57Bl/6 organoids, but not tumors, clustered together (Supporting Fig. S8B). Moreover, comparative analyses and cross-species integration of the tumoroid expression signatures with a previously published data set of 70 human HCCs, 13 CCAs, and 7 liver cancers of mixed HCC/CCA histology\textsuperscript{20} confirmed that both the HCC-like and CCA-like tumoroids closely reflected the transcriptome profile of respective authentic human cancers (Fig. 5F).

These results indicate that, despite their initial biliary phenotype, the genetic profile of the liver organoids significantly contributes to the morphology of resulting tumors in \textit{vivo}.

Discussion

CCA is the second-most common primary liver cancer. A rising incidence and pronounced resistance
to current treatment regimens emphasize the need for a better pathophysiological understanding, which is pivotal for the development of novel treatment approaches. Multiple studies have investigated the mutational landscape of CCA. To functionally address the role of potential cancer drivers during tumor development and maintenance, as well as to identify effective drug targets, histologically accurate and genetically flexible in vivo systems are needed. Organoid cultures offer unique opportunities to model CCA. Unlike other primary culture systems, such as fetal liver cells, organoids can be serially passaged in an untransformed state, cryopreserved, infinitely expanded, and genetically modified according to specific objectives of individual investigations. Importantly, organoid cultures offer the opportunity to minimize cost and time required for continuous animal husbandry and/or repeated isolations of primary cells, thus significantly accelerating the translation of scientific hypotheses into experimental approaches.

We demonstrate that genetic modifications can be rapidly introduced into liver organoid cultures by RNA interference or CRISPR/Cas9 technology. Co-introduction of antibiotic resistance genes facilitates the in vitro selection of efficiently targeted cell populations before transplantation. This feature is especially convenient when studying genetic alterations that do not convey a selective advantage to the emerging tumor and might otherwise be outgrown by nonmodified clones. Furthermore, compared with traditional transgenic models, this model can incorporate genetic alterations in multiple genes simultaneously, offering the genetic flexibility to model highly complex genetic interactions across diverse genomic loci.

Our work demonstrates that, in the context of mutant Kras, liver organoids derived from adult murine livers can give rise to tumors histologically matching CCA with 100% penetrance when injected orthotopically or s.c. in syngeneic animals. Unlike in transgenic approaches, in which poorly differentiated sarcomatoid tumors or overlaps to tumors of hepatocellular differentiation are frequently observed, more than 90% of CCA are moderately differentiated CCA (G2). Given the decisive importance of the tumor stroma in tumor development and therapy response, the observed distinct stromal reaction in the genetically modified organoid model closely recapitulates this hallmark feature of human CCA. Therefore, the model can be highly instrumental to study tumor–stroma interactions in the emerging field of stroma biology.

Liver tumorigenesis is considered a multistep process. Using expression profiling and array comparative genomic hybridization, we provide initial evidence for how the molecular profile of organoids undergoes gradual genetic changes, reflecting the key transforming events from cells that are WT or harbor latent cancer alleles, toward a "premalignant" in vitro activated phenotype following introduction of defined genetic alterations, until reaching the fully transformed state of malignant tumors.

Indeed, transcriptome alterations are most pronounced in tumors, likely due to the outgrowth of highly malignant clones under selective pressure, in combination with the acquisition of additional lesions during in vivo tumorigenesis. Similarly, the acquisition of pro-oncogenic copy number alterations can be detected during the progression from in vitro

FIG. 4. In vivo validation of liver cancer genes in the organoid model. (A) Murine organoids can be used to validate candidate cancer drivers in cholangiocarcinogenesis. Kras<sup>(2012)T24</sup>, p53<sup>(143)W12</sup> organoids were transduced with retroviruses expressing GFP-coupled shRenilla (control) or shPten (LMP<sub>shRenilla</sub> or LMP<sub>shPten</sub>) and transplanted into recipient mice. Loss of Pten significantly accelerates tumor development over controls, as assessed by tumor weight. (B) Genetically modified organoids give rise to predominantly moderately differentiated CCA. (C) Immunoblotting confirms loss of Pten in shPten-expressing tumor cell lines and up-regulation of downstream mediators (phosphorylated Akt). (D) Immunohistochemistry shows loss of Pten only in CCA and GFP-positive tumor cells, but not in the recipient-derived surrounding stroma. (E) Schematic of the sgRNA/shPten-CC vector that facilitates efficient genome editing in the CCA model using CRISPR/Cas9 technology. Kras<sup>(2012)T24</sup> liver organoids were transfected with the sgRNA/shPten-CC vector that simultaneously encodes for sgRNAs directed against the tumor suppressors p53 and Pten, the Cas9 enzyme, and Cre recombinase. Efficient cleavage of p53 and Pten by the sgRNA/shPten-CC vector in murine pre-injection organoids and tumors is detected by T7 endonuclease assays (left), and immunoblotting confirms loss of Pten protein and p53 protein expression in a tumor-derived CCA cell line (right). Protein extracts from NIH-3T3 cells serve as a control. Asterisks mark tumor-derived cell lines. (F) Immunohistochemistry on CRISPR/Cas9-generated tumors reveals hallmark features of CCA histology as described. Scale bar corresponds to 200 µm.

Abbreviations: Akt, thymoma viral proto-oncogene; G2, grade 2; G3, grade 3; and H&E, hematoxylin and eosin.
modified organoids to tumoroids. Our study highlights that the de novo introduction of driver oncogenes in combination with tumor suppressor gene loss is capable of introducing specific genomic changes, thus significantly altering the genetic landscape of liver organoids.

The murine liver organoids complement the human counterpart by enabling scientific work in genetically induced tumors that allow, among others, the study of vulnerabilities in the presence of defined combinations of genetic alterations that occur in large fractions of patients with CCA. In addition, considering the significant advances made in the field of tumor immunology, transplantable murine organoids allow us to study CCA in an immunocompetent and syngeneic environment. An additional benefit of our model is the simplicity with which the transition between in vitro and in vivo work can be accomplished. Re-transplantation of both 2D tumor-derived cell lines and tumoroids gives rise to cancers that recapitulate the histology of the primary tumors, which indicates that the tumor cell lines retain crucial features of corresponding parental tumors. In addition, 2D and 3D tumor-derived cell lines exhibit comparable susceptibility to targeted therapies. These results suggest that these cell lines are a competent and viable tool for functional in vitro studies, such as drug screening.

The cellular origin of liver cancer remains unresolved. Indeed, in experimental animal models, hepatocytes, as well as bipotent progenitor cells, can give rise to CCA and HCC in a context-dependent and genotype-dependent fashion. Liver organoid cultures are most likely initiated from cells that reside within the biliary epithelial compartment, as suggested by the expression of biliary markers and the inability of mature hepatocytes to contribute to organoid cultures under the described conditions. Despite their biliary phenotype, we were able to show that, in direct dependence on the oncogenic driver, organoids retain their plasticity and can be transformed into tumors that show a markedly different histology than CCA and more closely resemble HCC. Importantly, these tumors also exhibit expression profiles that cluster with human HCCs. Therefore, our genetically modified liver organoids can recapitulate the full morphological and molecular spectrum of primary human liver cancer.

In summary, we have shown that, depending on the genetic context, organoids can give rise to aggressive liver cancers that exhibit characteristics of human CCA as well as HCCs in immunocompetent mice. These tumors not only histologically resemble their human counterparts but also show similar expression profiles. Thus, organoid-based mouse models can serve as a scalable system to facilitate the rapid interrogation of putative cancer genes and vulnerabilities using RNA interference and CRISPR/Cas9 technology.

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REFERENCES


Supporting Figures

Supporting FIG. S1: EpCAM FACS gating strategy
EpCAM FACS on whole liver digests versus isolated liver organoid cell line. Abbreviations: EpCAM, epithelial cell adhesion molecule; FACS, fluorescence-activated cell sorting; SSC-A, side scatter area; SSC-W, side scatter width; FSC-A, forward scatter area.

Supporting FIG. S2: Abundant budding of organoids from tissue fragments
Murine liver digests and remaining tissue fragments were plated separately. Tissue fragments lead to accelerated and more abundant organoid development as compared to the cell suspensions.
Supporting FIG. S3: Cre-recombinase mediated activation of latent transgenic alleles

(A) Successful Cre-recombinase mediated excision of the transcriptional stop cassette within the Kras\textsuperscript{Is1G12D} allele and loss of the floxed p53 allele in murine organoids is confirmed by multiplex PCR\textsuperscript{(1)} using the following primers: y116 WT: TCC GAA TTC AGT GAC TAC AGA TG; y117 MT: CTA GCC ACC ATG GCT TGA GT; y118 common: ATG TCT TTC CCC AGC ACA GT. Presence of the p53 floxed allele 1F: CAC AAA AAC AGG TTA AAC CCA G; 1R AGC ACA TAG GAG GCA GAG AC; and Cre-mediated recombination of the lox sites (1F and 10R GAAGACAGAAAAGGGGAGGG) was tested. (B) Murine Kras\textsuperscript{Is1G12D/wt};p53\textsuperscript{lox/lox} liver organoids do not exhibit overt morphological changes upon Cre-recombination. Abbreviations: Kras, Kirsten rat sarcoma oncogene; bp, base pair; fl, floxed; lsl, lox-stop-lox; MT, mutant; PCR, polymerase chain reaction; WT, wild type.
Supporting FIG. S4: Gene Set Enrichment Analysis on wild type, preinjection and tumor derived organoids

(A) Gene sets activated during the progression from wild type to preinjection organoids.
(B) Gene sets activated during progression from preinjection organoids to tumor derived organoids (tumoroids).
Supporting FIG. S5: Gemcitabine treatment leads to a modest survival benefit in mice harboring murine CCAs
Kras\textsuperscript{G12D}:p53\textsuperscript{Δ/Δ} organoids were injected sq into recipient mice. Gemcitabine treatment (100 mg/kg bodyweight) was initiated when murine CCAs reached a diameter of >5 mm and led to a modest survival benefit (32 vs. 42 days; \( P = 0.027 \)). Abbreviations: CCA, cholangiocarcinoma; sq, subcutaneous.

Supporting FIG. S6: Relative stromal content is not altered by loss of Pten
As a surrogate for the amount of recipient derived stroma, CK19-negative area was determined on CK19 stained immunohistochemistry slides. Relative stromal content does not differ between CCAs with and without loss of Pten. Abbreviations: CCA, cholangiocarcinoma; CK19, cytokeratin 19; NS, not significant; Pten, phosphatase and tensin homolog.

Supporting FIG. S7: Relative stromal content is similar in orthotopic and subcutaneous CCAs
(A) Kras\textsuperscript{G12D}:p53\textsuperscript{Δ/Δ}:LMP\_shPten organoids give rise to orthotopic CCAs (macroscopic image) upon intrahepatic injection. GFP (transplanted cells) and CK19 (ductal differentiation) expression is confirmed by immunohistochemistry. (B) Orthotopic and subcutaneous CCAs do not differ in stromal content (as assessed by CK19-negative area). Abbreviations: CCA, cholangiocarcinoma; CK19, cytokeratin 19; GFP, green fluorescent protein; HE, hematoxylin and eosin; NS, not significant; sq, subcutaneous.
Supporting FIG. S8: Differential expression analysis of Myc expressing liver organoids

(A) sgRNA-mediated cleavage of Apc in Myc, shp53, sgApc organoids (preinjection, lane 2), and isolated tumoroids (lane 3) detected by T7 endonuclease assays. Lane 1: control genomic DNA. (B) Differential expression analysis of liver organoids from C57Bl/6J mice (wild type), C57Bl/6J liver organoids transduced with a Myc overexpression cassette, an shRNA against p53, and transiently transfected with an sgRNA targeting Apc prior to injection into recipient mice (preinjection), as well as tumoroids derived from a resulting tumor. Similar to our observations in CCA organoids (Fig. 2D), wild-type and preinjection organoids cluster together. (C) Treatment of 2D HCC-like tumor derived cell lines with selumetinib and BKM-120 at the same inhibitor concentrations used in Kras mutant cells lead to a significant, but only mild reduction in cell growth at 48 hours (see Fig. 3D for comparison). Abbreviations: 2D, 2-dimensional; Apc, adenomatous polyposis coli; CCA, cholangiocarcinoma; GFP, green fluorescent protein; Myc, myelocytomatosis oncogene; sgRNA, single guide RNA; shRNA, short hairpin RNA.

References for Supp. Fig. Legends
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**Supporting Information: Supplemental Materials and Methods**

### Subcutaneous and orthotopic cell transplantation

For subcutaneous (sq) injections, $0.5 \times 10^6$ organoids were resuspended in 50 µL of 50% Growth Factor Reduced Matrigel (Corning, NY) and injected subcutaneously into the rear flanks of recipient mice. 2D cell lines were resuspended in 50% Matrigel/PBS and injected. For orthotopic transplantation, a substernal 5-mm longitudinal incision was performed, the left liver lobe was exposed, and organoids were implanted using a 30-g Hamilton syringe. After retraction of the needle, the injection site was compressed with a sterile cotton swap, and the abdominal cavity was washed with 2 mL of sterile prewarmed water. The abdominal wall was closed layer-wise using absorbable sutures.

### Transfection and retroviral transduction of organoids

MSCV-based retroviruses (pMSCV-LTR-miR30-Puromycin-resistance-IRES-EGFP [LMP]\(^1\) and PGK-Cre\(^{ERT}\)-IRES-Neomycin\(^{Resistance}\) [PCIN]) were produced in Platinum-E retroviral packaging cells (Cell Biolabs, San Diego, CA), concentrated using Retro-X concentrator (Clontech, Mountain View, CA), and supplemented with polybrene (4 µg/mL) prior to transduction of organoids. ShRenilla.713 (shRenilla) and shPten.1522 (shPten) were described.\(^2\) sgApc was cloned into pX459 (Addgene, plasmid #48139). Transient transfections of organoids with pX459_sgApc and U6-sgp53-U6-sgPten-EFS-Cas9-P2A-Cre plasmid (sgp53/sgPten-CC)\(^3\) were performed using Lipofectamine2000 (ThermoFisher Scientific, Waltham, MA). Cas9-mediated DNA cleavage of p53 and Pten were verified using the T7 Endonuclease I EnGen Mutation Detection Kit (NEB, Ipswich, MA) according to the manufacturer's manual. PCR products were heteroduplex annealed and treated with Endonuclease T7.

### Guide RNA sequences:

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### Primers used in T7 Endonuclease Mutation Detection Assay

| T7 Mut PCR p53 fwd | GCCATCTTGGGTCCTGACTT |
| T7 Mut PCR p53 rev | CCCCCGAGATTTCAGACA |
| T7 Mut PCR Pten fwd | GAGCCATTTCATCTGCAG |
| T7 Mut PCR Pten rev | CTAGCCGAACACTCCCTAGG |
| T7 Mut PCR Apc fwd | GCCATCCCTTCACGTAG |
| T7 Mut PCR Apc rev | TTCCACTTTGGCATAGGC |

### Immunohistochemistry, immunofluorescence, Alcian Blue- and Sirius Red/Fast Green stains

Slides were deparaffinized and rehydrated. Sirius Red/Fast Green staining was performed using Sirius Red Solution (0.5 g Direct Red; Sigma-Aldrich, St Louis, MO) and 0.5 g Fast Green FCF (Sigma-Aldrich) in 500 mL Picric Acid-saturated solution 1.3% (Sigma-Aldrich).

For Alcian Blue staining (Serva Electrophoresis, Heidelberg, Germany), the deparaffinized slides were immersed in 3% acetic acid and stained in Fast Red for 30 seconds. Hematoxylin
and eosin (H&E) staining and immunohistochemistry were performed as described.\(^4\) For immunohistochemistry, we used the following primary antibodies: Abcam (Cambridge, UK): CK19, #ab133496 and GS, #ab73593; Cell Signaling Technology (Danvers, MA): GFP XP #2956, PTEN XP #9188s and CD31 #77699S; Santa Cruz Biotechnology (Dallas, TX): CK7, #sc-23876. The secondary biotin conjugated antibody (goat-anti-rabbit, #B-2770, Life Technologies, Carlsbad, CA) was diluted 1:250. For immunofluorescence, all primary antibodies were purchased from Santa Cruz Biotechnology and used at a 1:50 dilution: β-Catenin (#sc-7963), CK7 (#sc-23876), CK19 (#sc-33111), and Sox9 (#sc-20095).

**Immunoblotting**

Immunoblotting was performed as described.\(^4\) We used the following primary antibodies: Cell Signaling Technology (Danvers, MA): Akt Pan, #4691P, p-Akt (T308), #2965S, p44/42, #4695S, PTEN XP, #9188s, p-p44/42 (T202/Y204) #4376S (all 1:1000), Vinculin XP, #13901 (1:5000); Santa Cruz Biotechnology (Dallas, TX): p21 (#sc-471, 1:500); Leica Biosystems (Wetzlar, Germany): p53, #p53-CM5P-L (1:2000). Secondary antibodies (1:5000): goat-anti-mouse (Santa Cruz, #sc-2005) and goat-anti-rabbit (Cell Signaling Technology, #7074S). Cells were treated with 1 µg/mL doxorubicin for 60 minutes prior to blotting for p53.

**Gemcitabine treatment**

C57/Bl6 mice were injected sq with Kras\(^{G12D/wt}\); p53\(^{Δ/Δ}\) organoids and randomized upon detection of a tumor of 0.5 cm diameter into either a treatment (gemcitabine, n = 5, 100 mg/kg, intraperitoneally) or a placebo (n = 6, NaCl 0.9%) arm. Mice were followed by caliper measurements and harvested upon reaching endpoint criteria (tumor volume 1,400 mm\(^3\), poor health conditions).

**Detection of copy number alterations (CNAs) in murine organoids and tumoroids by comparative genomic hybridization (CGH)**

DNA from Kras\(^{G12D/wt}\); p53\(^{lox/lox}\) (wild type) Kras\(^{G12D/wt}\); p53\(^{Δ/Δ}\) (preinjection) and Kras\(^{G12D/wt}\); p53\(^{Δ/Δ}\) (tumoroid) murine liver organoids was isolated according to the manufacturer’s protocol (NucleoSpin Tissue; Macherey-Nagel, Düren, Germany), labeled, and hybridized to an Agilent 4 × 180k MicroArray (Agilent Technologies, Santa Clara, CA). For visualization of array CGH data, genomic copy number alterations were identified using the R package CGHcall\(^5\) applying default parameters followed by the definition of genomic copy number regions using the R package CGHregions.\(^6\) The called copy number regions were collapsed to three states (i.e., loss, normal, gain) and plotted along the genomic position next to ideograms of mouse chromosomes. CNAs that occurred in the genetically modified organoids or in tumoroids were identified in contrast to the genomic copy number profiles of parental wild-type organoids. The genomic locations of CNAs in genome version NCBI37/mm9 were translated to GRCm38/mm10 using LiftOver tool provided by the UCSC genome browser. Genes encoded on CNA regions were extracted from the Ensembl genome database using the R bioMaRt package.\(^7\) Segmented genomic copy number profiles generated from Affymetrix SNP 6.0 genotyping array data in the frame of the TCGA cholangiocarcinoma project were downloaded from the GDC data portal (https://portal.gdc.cancer.gov) and subjected to copy number calling using CGHcall and CGHregions. The alteration frequency of
gains and losses of genes that were detected in the mouse organoids was subsequently determined in the human data set.

**RNA sequencing**

RNA from Kras\(^{lslG12D/wt}\); p53\(^{lox/lox}\) (wild type organoids), Kras\(^{G12D/wt}\); p53\(^{∆/∆}\) ("preinjection" organoids) and tumor-derived Kras\(^{G12D/wt}\); p53\(^{∆/∆}\) ("tumoroids"), as well as Myc;shp53;sgApc (Supporting Fig. S7B, preinjection and tumoroid) and C57BL/6J murine liver organoids (Supporting Fig. S7B, wild type) (n = 3 replicates each), was isolated according to the manufacturer’s protocol (NucleoSpin RNA #740955, Macherey-Nagel). RNA integrity was confirmed on an Agilent 2100 Bioanalyzer. NEB Next Ultra RNA Library Prep Kit was used for nonstranded library preparation, and samples were sequenced on an Illumina HiSeq 4000.

Raw reads were filtered by removing adapter sequences, contamination, and low-quality reads. The reads were mapped to mouse genome reference sequence (GRC38m) using HISAT2 (hisat2-2.0.2-beta) followed by read summarization with featureCounts (subread-1.5.0-p1). All data analysis was performed using R programming language and related packages. Output matrix from featureCounts was used as input for Bioconductor package DESeq2 to perform differential expression analysis. Significance testing was performed using Wald Test statistics. Only genes with a fold change >2 were considered for further analyses. To visualize the data, Euclidean distance clustering was performed. All plots were generated using the MADE4 package. Gene Set Enrichment Analysis (GSEA) was performed using GSEA software provided by Broad Institutes [http://www.broad.mit.edu/gsea/]. Gene sets from the MSigDB database were tested and gene sets with a NOM P value <0.05 and FDR <0.25 were considered significantly enriched in an a priori defined set of genes. Integration of human data was performed as described using GSE15765.

**Determination of CK19-negative area**

CK19-negative area was determined by automatic thresholding using ImageJ (National Institutes of Health, USA). Five nonoverlapping low-magnification fields of view were considered per tumor.

**Cell Cycle Analysis**

Cells were plated at a density of 0.04 × 10\(^6\) cells per 12-well and treated with IC50 concentrations of selumetinib (3.36 µM; MedChem Express, Monmouth, NJ), BKM-120 (0.25 µM; MedChem Express, Monmouth, NJ), or a combination of both for 24 hours. Cells were harvested, washed once with PBS, and fixed with 70% EtOH at −20°C overnight. Thereafter, 1 mL Trisodium citrate (38 mM) was added, and samples were centrifuged for 5 minutes at 500g. The supernatant was discarded, the pellet was resuspended in PI staining solution (400 µL Trisodium citrate, 30 µL PI [2.5 g/L], 5 µL RNase A [10 mg/mL]), and incubated at 37°C for 20 minutes protected from light. The samples were analyzed by flow cytometry within 1 hour.
**Statistical analysis**

Experimental data were analyzed using GraphPad Prism software. 1-tailed $t$ test was used to compare cell growth (Fig. 1C) and tumor weights (Fig. 4A). We applied a 2-tailed $t$ test to compare CK19-negative area (Fig. 3C; Supporting Figs. S5, S6B). Log-rank (Mantel Cox) test was used to calculate differences in animal survival (Supporting Fig. S4). 1-way ANOVA with Post-Bonferroni’s Multiple Comparison Test was used in cell viability assays (Fig. 3D).


Generation of focal mutations and large genomic deletions in the pancreas using inducible in vivo genome editing


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† These authors contributed equally to this work.

Abstract
Beyond the nearly uniform presence of KRAS mutations, pancreatic cancer is increasingly recognized as a heterogeneous disease. Preclinical in vivo model systems exist, but with the advent of precision oncology, murine models with enhanced genetic flexibility are needed to functionally annotate genetic alterations found in the human malignancy. Here, we describe the generation of focal gene disruptions and large chromosomal deletions via inducible and pancreas-specific expression of Cas9 in adult mice. Experimental mice were derived on demand directly from genetically engineered embryonic stem cells, without the need for further intercrossing. To provide initial validation of our approach, we show that disruption of the E3 ubiquitin ligase Rbx1 accelerates KrasG12D-dependent tumourigenesis. Moreover, we demonstrate that this system can be used to rapidly interrogate the impact of complex cancer-associated alleles through the generation of a previously unstudied 1.2 megabase deletion surrounding the CDKN2A and CDKN2B tumour suppressors. Thus, our approach is capable of reproducibly generating biologic and precise loss of large chromosomal fragments that, in conjunction with mutant Kras, leads to development of pancreatic ductal adenocarcinoma with full penetrance.

Introduction
Patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) face a devastating 5-year survival rate of approximately 8% (1). Genetically engineered mouse models (GEMMs) of PDAC have both improved our understanding of the pathology of this deadly disease and been used as preclinical models to evaluate new therapeutic strategies. Existing models reflect predominantly only simple cancer genotypes (e.g. KrasG12D/lp53+/−) (2), yet it is increasingly evident that accurately depicting the many distinct subgroups of human PDAC will require more complex genetic approaches. Indeed, many cancer-associated genetic alterations, including focal mutations and large-scale deletions, amplifications and fusions identified through sequencing studies, remain to be tested functionally in order to delineate their impact on cancer progression and therapeutic...
response. For instance, large genomic deletions encompassing the CIN24A/28 locus are among the most common alterations in PDAC (5-6) with distinct therapeutic implications (9,10). Although loss of CIN24A is undoubtedly a critical driver, these deletions include many surrounding loci which may contribute to the oncogenicity of this genomic event. The generation of homeo-
geny genomic deletions in mice has been particularly labor in-
tensive in traditional Cre/Lox-based approaches, which require the integration of two loci sites by homologous recombination, and subsequent repeated intercrossing to attain homeogeneity. In addition, recombination efficiency decreases with genomic distance, making large homeogony deletions particularly chal-
 lenging to model (9-10). As such, large deletions have remained underexplored in in vivo models and more flexible systems are needed to model such complex alleles.

Previously, we described an embryonic stem cell (ESC)-based GEMM (GEMM-ESC) that enables the rapid generation of genetical complex transgenic animals directly from ESCs. In our earlier work, we generated ESC lines with pancreatic-specific ex-
pression of Tet-regulatable, EGFP-coupled short-hairpin RNAs and a mutant Krüten Rat Sarcoma oncogene (Kras) G12D (11).

In this study, we adapted our system to exploit an inducible in vivo CRISPR/Cas9 approach (CRISPRN) (12) to study pancreatic carcinogenesis and sought to assess the capabilities of this novel platform to interrogate the impact of both single gene trans-
ditions and large genomic deletions. We show that loss of func-
tion of the candidate cancer gene Ring finger protein 43 (Rnf43) accelerates mutant Kras-driven tumorigenesis, and that disparate homeosome deletions of the extended CIN24A/28 locus can be modeled with full penetrance.

Materials and methods

Single guide RNA design, cloning and validation

Single guide RNAs (sgRNAs) were designed using CRISPOR software (13) and inserted into the psp95 vector (Addgene plasmid #2496) (Efficient in site genome editing was confirmed by T7 endonuclease I assay (T7-EN), New England Biolabs, MA) according to the manufacturer’s protocol. For integration into the K16-ESC, U6-EGFP RNA Fragments were subcloned into the NotI sites of the CRISPN targeting vector (Addgene plasmid #219). Primers used in T7-EN endonuclease I assays and for deletion detection are listed in Table S1 (available at Carcinogenesis Online).

ESC culture, recombination-mediated cassette exchange and functional characterization

ESCs were maintained in M1 - leukemia inhibiting factor (LMIF) (LIF, Sigma, Munich, Germany) and an embryonic medium containingembryonic stem cell growth factor (EGF) (100 ng/mL, Lonza, Basel, Switzerland) and an AMAXA Nuclefect II (Lonza, Basel, Switzerland) according to the manufacturer’s protocol. Following fbroblast selection (1-10 days), correct targeting was verified by PCR as described (15). Prior to further experiments, embry-
tity (day 0) embryonic mouse models (ESCs) were extracted cloned by T7 endonuclease I assay, and efficient inducible genome editing was tested in vivo upon electroporation of the Cre-recombinase (High Con-
yency, #605108) into targeted K16-ESC-ESC. Followed by a 48-hour dose treatment, mKate2 and EGFP expression was analyzed on a BD FACScalibur (BD Biosciences, San Jose, CA).

Animal experiments

All animal experiments were performed according to protocols approved by the local authorities (The Lower Saxony State Office for Consumer Protection and Food Safety, LAVES). The generation of the K16-KO-ESC line has been described elsewhere (13). In brief, the K16-ESC line’s chromosomal mice were generated by morula injection at the Max Planck Institute for Biophysical Chemistry, Göttingen, Germany. Unless indicated otherwise, K16-KO-ESC and -EGFP mice were involved to dose-enriched food (KIS mg/kg, Altromin, Lage, Germany) for 2 days prior to birth for a total of 16 days. All K16-KO-ESC mice were involved in a 3,100 mg/kg daily doses were involved to dose-enriched food (KIS mg/kg, Altromin, Lage, Germany) for 2 days prior to birth for a total of 16 days. All K16-KO-ESC mice were involved in a 3,100 mg/kg daily doses were involved to dose-enriched food (KIS mg/kg, Altromin, Lage, Germany) for 2 days prior to birth for a total of 16 days.

Hematoxylin & eosin (H&E), alcin blue staining, immunohistochemistry and western blot

H&E, alcin blue staining and immunohistochemistry were performed as previously described (14). The following antibodies were used for immunohistochemistry: anti-SF7 (1:1000), K16-ESC (1:200); ZEB1 (1:200), ZEB2 (1:1000), APE1 (1:200), P21 (1:1000), XBP1 (1:500). All immunohistochemistry and western blotting conditions were used for western blot.

Secondary antibodies goat anti-rabbit IgG (1:500), Life techno-
ologies, Gaithersburg, MD and goat anti-mouse IgG (1:500) (1:1000, 1:500) (1:500, 1:500, Santa Cruz Biotechnology). DAPI was used for immunohistocchemistry and western blot, respectively.

Cell line

Tumour tissue was minced and enzymatically digested for 60 minutes in digestion solution containing 0.1 mg/mL collagenase IV (08583, Sigma, St. Louis, MO) and 0.1 mg/mL DNase (DN25815, Sigma, St. Louis) in Hanks’s (with Ca2+/Mg2+) (1:5455-846 Gibco, Life Technologies). The cell suspension was filtered through a 70 mm cell strainer, and cells were cultured in Dulbecco’s modified Eagle’s medium (10% fetal bovine serum + penicillin/ streptomycin) for a minimum of seven passages. The immortalized cancer cell line 3DPC2 was a gift from Jennifer Metten and the K16-ESC line was a gift from Ronald Malekhim. Autoradiography was performed by generating PCR for the transgenic allele (PCR tested in May 2018 and June 2018, respectively).

Array comparative genomic hybridization

Array comparative genomic hybridization (aCGH) was performed as previously described (16). Targeting vectors were co-transfected with an FBS recombinase expressing vector (16) into K16-ESC-ESC cells using the NioVE Embryonic Stem Cell Nucleofection Kit (V977-180, Lonza, Basel, Switzerland) and an AMAXA Nuclefect II (Lonza, Basel, Switzerland) according to the manufacturer’s protocol. Following fbroblast selection (1-10 days), correct targeting was verified by PCR as described (15). Prior to further experiments, embry-
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yency, #605108) into targeted K16-ESC-ESC. Followed by a 48-hour dose treatment, mKate2 and EGFP expression was analyzed on a BD FACScalibur (BD Biosciences, San Jose, CA).
Human genomic copy number data

The GISTIC calls of the genomic copy number data of the human pancreatic adenocarcinoma data set provided by The Cancer Genome Atlas consortium were downloaded from cBioPortal (http://www.cbioportal.org). Data set (3), read tag per sample 2S18, e = 118. For the calculation of copy number alteration frequencies, only GISTIC calls of -2, i.e., homozygous deletion and +1, i.e., high-level amplification were used. For visualization, the genomics positions of genes were retrieved from Ensembl (REGLIST.12) using functions of the bamtools R package (18). Plotting was conducted in R using standard functions.

Statistics and models

Survival curves were generated and analyzed using GraphPad prism 7 software (GraphPad Software, San Diego) and Mantel-Cox test. Flow cytometry data were analyzed using Flowjo (Treestar Inc, OR, USA), and statistical significance was calculated by Student's t-test. All images were cropped in Adobe Photoshop and assembled in Adobe Illustrator (Adobe, San Jose). Fluorescence images were merged using ImageJ (139, NIH, Bethesda, MD). The low-magnification images shown in Figure 4E were assembled from multiple images taken by the ×4 ×4 map function in ScanScope pro imaging software on a Zeiss Axiolims microscope (Zeiss, Oberkocher, Germany). Sequenced PCR products from deletion tumour cell lines were aligned against the expected sequences using GenBank (29). Alignment results were visualized using MUSCLE (46).

Results

iCRISPR drives efficient gene editing in ECSC and in the pancreas

We previously published a GIMM-ESC platform capable of rapidly generating multiple allelic pancreatic cancer prone mice (11,22) termed KI-RIK (ECSC) that harbour a latent KrasG12D mutant (25), a pancreas-specific Cre-LoxP driver, Cre-recombinase (27) and a CAMOS-IRES-IRES-tdEYFP-mKate2 allele (CAMS-tdEYFP) (25) to co-express a reverse tetracycline transactivator (rtTA3) and a red fluorescent reporter, mKate2, upon Cre-mediated recombination. A Coll/2a homing cassette (CICD) downstream of the Col2a1 gene, that integrates genetic elements in vitro by RMCE accounts for the genetic flexibility of the system (29) (Figure 1A). To enable inducible genome editing, we utilized the GSCIR-mediated plasmid that encodes a TREG-driven EFGP-IRES-tdEYFP cassette and an sgRNA under the control of a constitutive U6 promoter (Figure 1B) (22). This iCRISPR system has been successfully used for gene editing in XIG-ESC in vitro as well as in the intestine of adult mice (21,28).

To assess whether the TREG promoter would drive sufficient Cas9 expression in our KI-RIK-ECSC to achieve efficient gene editing, we integrated the GSCIR vector harbouring a control sgRNA targeting a non-germ region on mouse chromosome 8 (g8CB2-B) (22). Gene editing from the GSCIR vector has been observed in targeted ECSCs in the absence of dox (12). Therefore, we first tested the targeted dox-responsive KI-RIK-ESC clones for the presence of insertions and deletions (inBetas) by T7 endonuclease I assay. Editing of the target region was found in 7/12 ESC clones tested (Figure 1C). For further in vitro and in vivo experiments, only non-mutated ESC clones were chosen. To assess induction of EGFP-coupled Cas9 by the TREG promoter in vitro following genetic integration, we induced expression of rtTA3 (and mKate2), by nucleofection of Cre-recombinase encoding plasmid (pG7GCre). Forty eight hours after addition of dox to the cell culture medium, 72% of mKate2-positive ESCs expressed EGFP as an indicator of Cas9 induction (Figure 2, Supplementary Figure 1, available at Carcinogenesis Online), and 17 always confirmed the presence of indels on chromosome 8 (Figure 1D).

Next, we generated KCrRIK-sgCR8 mice by morula injection and, to determine the extent and pattern of TREG-induced EGFP-coupled Cas9 expression within the adult pancreas, kept two 3-week-old animals on dox-food for 7 days before sacrifice. The TREG promoter led to mosaic pancreatic EGFP expression (Figure 1F; G). IHC EGFP-negative cells marked with an asterisk). To functionally confirm the efficiency and organ specificity of CSIR-mediated Cas9 genome editing, we comparatively performed 77 endonuclease I assays on genomic DNA extracted from the pancreas and from the tail of these mice. CRISP cleavage was readily detected in the pancreas, but not in the corresponding tail DNA, confirming the spatial control of TREG driven Cas9 activation, and the ‘tightness’ of the TREG promoter (Figure 1G).

RnF3 edited pancreata exhibit acinar-to-ductal metaplasia and pancreatic intraepithelial neoplasias but not intraductal papillary mucinous neoplasms

Loss-of-function mutations in RnF3 have been described in multiple cancers, including gastric cancer (21,32), colorectal cancer (23,34) and cholangiocarcinoma (59). In the pancreas, both intraductal papillary mucinous neoplasms (IPMN) and PDAC harbour RnF3 mutations in 14–16% and 5%, respectively (5,36,37). RnF3 is a ubiquitin E3 ligase that suppresses Wnt/β-catenin signaling by promoting the degradation of Wnt ligand receptor complex (19,39). To assess the utility of the KC-RIK-sgRnF3 system for testing candidate cancer genes, we inserted two different sgRNAs (g8CB2-A and g8CB2-B) targeting RnF3 into the CICD locus of KI-RIK ESCs by RMCE and screened ESC clones to exclude those with dox-independent target site alterations (Supplementary Figure 3A and 3B, available at Carcinogenesis Online). We confirmed efficient dox-dependent Cas9 expression and RnF3 editing (Supplementary Figure 2C and D), available at Carcinogenesis Online and chose one clonal ESC line per sgRNA (KCI-RK-sgRnF3-A and -sgRnF3-B) for further experiments.

Experimental cohorts of KC-RIK-sgRnF3-A and -sgRnF3-B derived mice were generated by morula injection. KCI-RK-sgRnF3 mice served as a control group. Pancreatic expression of Cas9 was initiated by feeding a dox-enriched diet for 14 days, beginning 7 days prior to birth. Three mice were randomly selected from the KC-RIK-sgRnF3-A and -sgRnF3-B cohorts and harvested after 8–10 weeks for histological evaluation. The remaining mice were allowed to thrive until meeting endpoint criteria. In 8–10 week-old KC-RIK-sgRnF3 mice, we observed an admixture of normal pancreatic parenchyma, acinar-to-ductal metaplasia and early pancreatic intraepithelial neoplasias (PanINs) (Supplementary Figure 2E, available at Carcinogenesis Online), overall similar to the pancreata from CR8 mice. Thus, at this stage, the histological changes are mainly attributable to expression of mutant Kras. Similar to the human counterparts, lesions corresponding to acinar-to-ductal metaplasia and PanINs were surrounded by a desmoplastic inflammatory stroma. mKate2 expression indicated efficient recombination in the exocrine pancreas and CK19 positivity confirmed ductal differentiation in the neoplastic lesion. The stromal compartment stained negative for mKate2, suggesting that it was not derived from recombined pancreatic progenitor cells undergoing epithelial-to-mesenchymal transition (Supplementary Figure 2F, available at Carcinogenesis Online).
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Figure 1. (A) Schematic of the relevant alleles in the KC-RKO-EGFP; the pancreas-specific Pdx1-Cre, the heterozygous Foxn4<sup>+/−</sup> model within the endogenous Foxn4 locus, the Collet locus harboring the Collet1 hemizygous cassette (CHC) with FR sites for recombination-mediated cassette exchange (RMCE), and the CAS9-RNase within the RNaseA locus. (B) Schematic of the targeting vector containing an sgRNAs under the control of the constitutive U6 promoter and FRT-DDEG-FRT-DDEG cassette, DDEG-encoding Cas9<sup>ΔN1014</sup> for RMCE-mediated integration. (C) Following RMCE-mediated integration of the targeting construct, E1C clones were rescued for dexamethasone (dex)-inducible mKate2 expression. mKate2<sup>+</sup> clones were harvested and induced with dexamethasone for 48 hours. Negative control: untargeted KC-RKO-EGFP; positive control: KRPc<sub>β</sub>3 cells edited with pPAX-sgEGFP. (D) KRPc<sub>β</sub>3 cells were harvested and induced with dexamethasone for 48 hours. Negative control: untargeted KC-RKO-EGFP; positive control: KRPc<sub>β</sub>3 cells edited with pPAX-sgEGFP. (E) Expression of EGFP reporter in KC-RKO-sgEGFP E1C clones after rescue with pPAX-DRE and 48 hours of dexamethasone treatment. Negative control: untargeted KC-RKO-EGFP; positive control: KRPc<sub>β</sub>3 cells edited with pPAX-sgEGFP. (F) Fluorescence image, immunohistochemistry for mKate2 and EGFP and H&E stain on KC-RKO-sgEGFP pancreata. Top right corner of the EGFP signal is displaced into the region marked with an asterisk. (G) T7 endonuclease I assay on genomic DNA from pancreas and corresponding tail DNA from KC-RKO-sgEGFP mice confirms genome editing in the pancreas but not in the tail. Negative control: untargeted KC-RKO-EGFP; positive control: KC-RKO-sgEGFP after rescue with pPAX-DRE and dexamethasone treatment.
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Online). Despite the recurrent loss of function mutations in K/R4 in human IPMN specimens, we did not detect larger cystic lesions resembling IPMNs in the KC-K/R4-K/R mice. CRISPR-mediated disruption of Rnfl3 accelerates neoplasia development and shortens survival of KC-K/R4-K/R mice

KC-K/R4-K/R mice exhibited a moderately reduced average median survival of 228 days compared with control KC-K/R4-K/R mice (median survival 303 days, F = 0.008; Barth KC-K/R4-K/R and KC-K/R4-K/R-A mice individually had shorter life spans compared with the controls, though only KC-K/R4-K/R-A mice formally reached statistical significance (214 days, F = 0.016; 246 days, F = 0.073, respectively; Figure 2A). Mice that reached endpoint criteria during the observation period presented with a fibrotic and largely remodeled pancreatic background neoplastic ductal lesions surrounded by a dense desmoplastic stroma (Figure 2E, top row). Cleavage of the target region was confirmed by T7 endonuclease I array in pancreatic biopsies (Figure 2B). Despite abundant desmoplasia, mean level frequency across 19 pancreatic biopsies reached 34.7% as determined by inference of CRISPR Delta (CRISPR) analysis (Figure 2A) (Figure 2C). Four of the 14 KC-K/R4-K/R mice (28.6%, Figure 2D) at risk showed poorly to moderately differentiated invasive PDAC upon histological examination after harvest (Supplementary Figure 3A, available at Carcinogenesis Online). In the KC-K/R4-K/R mouse, the frequency of invasive PDAC (representative example in Figure 2B, bottom row) was higher compared to the KC-K/R4-K/R-A group (sgrfl3-A: 57%, sgrfl3-B: 75%; Figure 2D). Markers of proliferation, apoptosis, stromal content and vascularization did not differ significantly between Rnfl3-edited pancreata and controls (Supplementary Figure 3B, available at Carcinogenesis Online). To further assess how the editing percentage predicted by ICE analysis correlates with the relative proportion of P53/Cre recombinant, mK/R4 positive cells (cells at risk for Rnfl3 editing) in pancreatic tumors, we stained PDAC-bearing KC-K/R4-K/R-K/R4 pancreata for mK/R4. In addition, we performed ICE analysis in consecutive tissue sections. Mean mK/R4 positive area was 49.50% and corresponding ICE analysis indicated an editing efficiency of 35.89% (Supplementary Figure 3H, available at Carcinogenesis Online).

These results suggest that Rnfl3 is edited at high frequency in pancreata of experimental mice and, in the presence of mutant K/R4, accelerates malignant progression in vivo.

Generation of large homozygous chromosomal deletions by dual sgRNA expression in vitro

Large genomic deletions are frequent events in human cancers. The INK4A/ARF locus is located on human chromosome 9 in a genomic region that is recurrently homozygously deleted in PDAC and other cancer entities. Analysis of The Cancer Genome Atlas provisional data (http://cancergenome.nih.gov) on 133 human pancreatic carcinoma specimens reveals that, in addition to the INK4A/ARF genes, several neighboring genes are co-deleted at high frequency (Figure 3A). This region is largely syntenic between mice and humans and is located on chromosome 4 in mice (Figure 3B). The size of the genomic deletion varies between patients but frequently exceeds one megabase (Mb) (Figure 3A).

In vivo modelling of homozygous deletions using CassA technology is particularly labour intensive and requires the genomic presence of four individual lox sites. CRISPR/CassA technology complements existing genome editing techniques and can be applied to generate deletions by tandem sgRNA expression. However, deletion frequency is inversely correlated with deletion size, and homozygous deletions exceeding 1 Mb are a particularly rare event (69). Unlike the focal genome editing that can be achieved in electroporation- or lentiviral-based systems, our inducible model is active throughout the entire pancreas. Therefore, we hypothesized that our P53/Cre-expressing KC-K/R4 model may be particularly well suited as an experimental system for the generation of large homozygous deletions. Considering the high deletion frequency of the genomic region flanking the INK4A/ARF locus in pancreatic cancer, we generated sgRNAs against target sites separated by ~1.5 Mb spanning a cluster of more than a dozen genes including Ink4a, Arf, Dmrt1, Mtyp and the interferon-I cluster (Figure 3B). The target sites were located in non-coding regions upstream of Ink4a (sgrfl3-A) and downstream of Dmrt1 (sgrfl3-B), respectively. Both sgRNAs were inserted into the sgrfl3 targeting vector (Figure 3C).

TREX-Independent Cas9 expression prior to RMCE leads to cleavage of either the sgrfl3-A, the sgrfl3-B or both the sgrfl3-A and sgrfl3-B targeting sites in a subset of targetted and hypermethylated EIC clones (42.9%, n = 16, Supplementary Figure 4A and B, available at Carcinogenesis Online). Apart from chromosomal deletions following two double strand breaks, non-homologous end joining repair of each double strand break without deletion of the intervening segment is an alternative outcome of dual guide RNA expression. Supporting the assumption that local editing of an individual sgRNA target site is a more frequent event than the excision of the entire chromosomal region that requires simultaneous DNA cleavage of both guide RNA target sites, deletion detection PCR was negative in all screened clones (Figure 3D).

We chose two clonets that did not exhibit cleavage at either of the two target sites for subsequent in vitro analysis. Following Cre-mediated activation of rtTA3 and induction of Cas9 expression by dox treatment for 48 hours, deletion-specific PCR analysis indicated the generation of deletions in both clones (Figure 3E). Sanger sequencing of the PCR product confirmed that the region upstream of sgrfl3-A was fused to the region downstream of the sgrfl3-B target site, resulting in the genomic excision of ~1.2 Mb fragment in vitro (Figure 3F).

KC-K/R4 mice develop PDAC due to large chromosomal deletions on chromosome 4 with 100% penetrance

Next, we generated mice from KC-K/R4-sgrfl3-ARF EICs via morula injection. Starting at 3 weeks of age, mice were fed a diet-containing diet for 21 days to induce Cas9 expression. Median survival was 40 days (Figure 4A), and all mice succumbed to histologically confirmed PDAC of poor to moderate differentiation (Figure 4A). Supplementary Figure 4C, available at Carcinogenesis Online). Notably, several tumour foci could be detected throughout the pancreas suggesting that malignant transformation occurred not only at a single, but at multiple sites within the organs (Figure 4D). The presence of the deletion was confirmed by PCR on genomic tumour DNA (Figure 4B, top row). To determine whether loss of the extended Ink4a/Arf locus was a heterozygous or a homozygous event, we established cell lines from individual tumours and propagated them for at least seven passages prior to subsequent analysis in order to reduce contamination with non-tumour cells. In all tumour-derived cell lines tested (n = 8), deletion PCR confirmed the sustained presence of the deletion (Figure 4B, middle row), whereas inefficient
Figure 2: Intrapancreatic disruption of Kras in the presence of mutant Ras leads to reduced survival. (A) Kaplan–Meier curves of Kras−/−xtgK14-Cre+ (A and B) and −/−xtgK14 mice editing of Kras leads to reduced aggregate survival of experimental mice (p = 0.002) compared with controls. Kaplan–Meier curves of Kras−/−xtgK14-Cre+ (A and B) mice versus −/−xtgK14 and Kras−/−xtgK14-Cre−/− mice exhibit a reduced survival (p = 0.0003 and p = 0.0087, respectively). (A) sgRNA1-A and −/−xtgK14 n = 14; sgRNA2 (B) and −/−xtgK14 n = 14. (B) Pancreata at the time of harvest after reaching endpoint criteria. Genomic editing of the targeted loci was confirmed by T7 endonuclease I assay (sgRNA1-A, −/−xtgK14-Cre−/− n = 14, sgRNA2 (B) and −/−xtgK14-Cre−/− n = 14). Representative examples shown. Negative control: untagged Kras−/−xtgK14-Cre−/−; positive control: Kras−/−xtgK14-Cre−/− expressing either sgRNA1 and −/−xtgK14-Cre−/− or −/−xtgK14-Cre−/− after manipulation with pFKE Cre and doxycyclin treatment. (C) Mean multifocal frequency in pancreatic bursa n = 14 and tail DNA n = 8 reached 34.7% and 1%, respectively, as determined by H&E analysis. (D) Frequency of histologically confirmed invasive PDAC assessed in a representative cross section of unimplanted pancreas from Kras−/−xtgK14-Cre+ (A), Kras−/−xtgK14-Cre−/− (B) and Kras−/−xtgK14-Cre−/− mice. (E) H&E staining and immunohistochemistry for markers and CK19 on two different Kras−/−xtgK14-Cre+ pancreata. Top row: neoplastic ductal structures surrounded by stellate-negative stroma, resembling human PDA. Bottom row: poorly to moderately differentiated invasive PDAC.
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Figure 2. CRISP9 mediated generation of a 1.2 Mb deletion in ESECs. (A) Schematic of the genomic region surrounding the ITGA6/ITGB7 genes on human chromosome 9. For each gene, the frequency of homologous deletions at 3+ fold gain is indicated in red and green vertical bars, respectively. (B) Schematic of the in vivo ablation on chromosome 4 in mice. (C) Schematic of the targeting vector containing two sgRNAs in tandem and a TR236-driven ECPI-REI-Cas9. (D) 1.2 Mb deletion in the dysplasia (left) and control (right) ESECs. Deletions detected by FISH using primers located 5’ of the ITGA6 and 7’ of the ITGB7 gene. Negative control: untargeted EC2-RK-EGFP; positive control: KRPC cells edited with pSpCas9-BB(A) and pSpCas9-BB(B). (E) Following electroporation of ESECs, immuno- and dye treatment for 48 hours, genomic deletions are readily detected by probed ecps. Negative control: untargeted EC2-RK-EGFP; positive control: KRPC cells edited with pSpCas9-BB(A) and pSpCas9-BB(B). (F) Target sequencing of the ECPS product confirms fusion of the genomic region upstream of Park1 (shown in green) to the region downstream of Dorset (shown in red) using the reverse primer for primer 9.
Figure 4. In vivo generation of an organ-specific CRISPR-Cas9-mediated homologous large genomic deletion surrounding the Ink4a/Arf locus. (A) Survival curve of KC-JE-\(\Delta\)AR mice. X-axis indicates time after start of dosing treatment. (B) PCR confirms the presence of the deletion in tumour DNA from eight random KC-JE-\(\Delta\)AR mice, as well as in tumour-derived cell lines passaged for at least seven times. The Jhakar/\(\Delta\)AR locus cannot be efficiently PCR-amplified in all cell lines tested, indicating its homogeny is due to homologous recombination. (C) Southern blot analysis revealed banding patterns consistent with the injected target site, indicating the presence of multiple tumour foci within the pancreas. (D) H&E staining of tumour sections from Ink4a/Arf-deficient mice with and without 4-OHT treatment. (E) Immunohistochemistry for Ki67 and CK19 in tumour sections from Ink4a/Arf-deficient mice with and without 4-OHT treatment.
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PCR amplification of Cdcna2 on genomic DNA indicated a homozygous loss (Figure 4B, bottom row). In addition, expression of Mtap, a gene adjacent to the Pdx1/Asf1 locus, was lost as assessed by immunostaining (Supplementary Figure 4C, available at Carcinogenesis Online). Sanger sequencing of the PCR product spanning the deletion site confirmed the presence of the fusion event. Size variations of the PCR products are likely a consequence of variations in the exact fusion location caused by differences during the DNA repair process (Supplementary Figure 4D, available at Carcinogenesis Online). Successful PCR-based amplification across the fusion junction, however, neither invariably rules out the loss of larger chromosomal fragments nor large chromosomal insertions on the second allele. Therefore, to assess whether the size of the deletion on both alleles reproducibly corresponds to the expected target sites of the two guide RNAs, we performed AGO1 on our eight individually derived tumour cell lines. These arrays confirmed that on both alleles, the deletion corresponds to the intended target sites on chromosome 6, spanning the 2.1 Mb starting upstream the Ifnat1 gene and extending to the region downstream of the Deut1 gene (Figure 4C, Supplementary Figure 5, available at Carcinogenesis Online). AGO1 further revealed that, except for the engineered deletion on chromosome 6, each tumour exhibited a unique profile with no recurrent additional events and only small chromosomal gains and losses, reflective of an overall low genomic instability commonly observed in murine tumours (Supplementary Figure 4, available at Carcinogenesis Online).

Considering that loss of Ink4b/E6b is a frequent event also in other cancer entities apart from pancreatic cancer, we reasoned that potential leakiness of our system might lead to malignant transformation within other organ systems. Both the liver and the long were screened for neoplastic lesions, but no macroscopic positive nodules could be detected outside of the pancreas, thereby validating the robust organ specificity of our approach (Supplementary Figure 7, available at Carcinogenesis Online). Thus, the GEMM-ESC-based KG-AK tandem guide RNA approach is a highly tractable tool to efficiently model large homozygous deletions with full penetrance in vivo.

Discussion

GEMM-ESC-based murine models are a rapid mouse modelling platform that combines the advantages of traditional transgenic approaches with great genetic and functional flexibility. We have previously incorporated inducible RNA interference technology into GEMM-ESC models to study the consequences of loss or re-expression of cancer-related genes in FASAM (11). In recent work, we used this model to delineate that the switch/suicide nonmerging complex member Atf6a functions as a potent regulator of Insr-induced changes in acinar cell identity (25).

CRISPR/Cas9 technology is a potent tool for genome editing applications owing to the flexibility with which it can be adapted to different germline targets and delivery systems (31). Unlike the knockdown phenotypes generated by RNAS, CRISPR/Cas9-mediated genome editing can select for a complete loss-of-function phenotype in mice when studying cancer genes. Here, we demonstrate that transgenic CRISPR technology in the pancreas serves as a powerful tool not only to validate novel cancer genes, but also to generate large homogenous chromosomal deletions with high penetrance and reliable organ-specificity in vivo. The use of CRISPR in genetically engineered EGC offers multiple advantages: The dual reporter system facilitates the tracing of relevant cell populations in vivo. In the presence of dose, the green fluorescent reporter DsRed is expressed as a surrogate for Cas9 induction. As the Pdx1 promoter-driven Cre recombines not only activates the mutant Kras allele but also drives a Kras-coding CAGS-KRas cell-specific for neoplastic transformation can be readily detected amongst non-pancreatic tissues such as intestinal cells or immune cell infiltrates. Timed induction of Cas9 expression and administration of a dox-enriched diet facilitates genome editing at various steps of pancreatic development or malignant transformation, and temporally uncouples Cas9-induced secondary genetic events from activation of mutant Kras. In addition, transient Cas9 expression by dox removal likely reduces off-target effects of the CRISPR/Cas9 system.

In this work, we demonstrate how transgenic CRISPR technology can be utilized to functionally investigate putative cancer driver genes in vivo. Recent data indicate that the RING finger protein Rbf1 has tumour suppressive functions in experimental models of gastric cancer and colorectal cancer (32). Loss-of-function mutations in RNF41 occur in 5% of pancreatic cancer specimens (5). In our mouse model, pancreatic loss of Rbf1 cooperates with mutant Kras to accelerate tumorigenesis and moderately reduces overall survival. RNF41 is also mutated in 14-35% of human (IPMNs) (33-37), a group of pancreatic cystic lesions at risk for neoplastic transformation. Notably, in the murine system, we histologically did not detect the typical cystic appearance of IPMNs, suggesting that in our model mutant Kras/Rn41-mediated PDAC development proceeds through precursor lesions such as acinar-to-ductal metaplasia/PanIN. Although the majority of Rbf1-edited pancreata harboured invasive PDAC, a subset of mice across all experimental groups exhibited a fibrotic and largely remodelled pancreas with PanIN lesions surrounded by dense stroma, consistent with reaching endpoint criteria. Massive remodelling can lead to a shortage of functional pancreatic tissue, resulting in pancreatic exocrine insufficiency and may be cause of non-tumour-related death in non-PDAC-bearing mice.

Synchronous expression of two sgRNAs targeting two chromosomal loci can result in the relatively rare event that the interesting DNA segment is deleted. Indeed, random CRISPR/Cas9 approaches have been used to create gene fusions in vitro and in vivo in the lung (42,43), the intestine (44) and in the liver (45). Most of these fusions, however, are heterogeneous and large homozygous deletions are particularly challenging to model in vitro because they require the bi-allelic deletion of two large genomic fragments. Recently, Carver et al. (46) assessed the efficiency with which genomic deletions are generated by introduction of two guide RNAs in vitro and experimentally confirmed a strong inverse relationship between the deletion size and frequency in more than 500 individual clonal lines harbouring one of three pairs of sgRNAs with intervening DNA segments between 70 kb and 1.1 Mb; no homozygous deletion was detectable in any of the clones (46).

Using transgenic and inducible CRISPR technology expressed from the CHC locus, we have shown that large homozygous deletion of more than 1 Mb surrounding the Ink4b/Arf locus can be generated and lead to tumour development with complete penetrance. Experimental evidence indicates that efficient deletion of DNA fragments using CRISPR/Cas9 is sensitive to sgRNA/Cas9 dose (47). In our system, the robust and timed Cas9 expression from a defined genomic locus reliably secures the controlled delivery of Cas9 and sgDNA without the risk of uncontrolled genome editing caused by continuous Cas9 expression. Multiple distinct tumour foci within the pancreas of experimental mice imply that numerous successful fusion events occurred. The
Ptf1a-Cre activator the CRISPR system in the vast majority of exocrine pancreatic cells. Therefore, although the generation of a large chromosomal deletion may be a rare genetic event at the cellular level, the sheer abundance of targetable cells and the robust and timed expression of Cas9 permits the reliable manifestation of the fusion phenotype, indicating that our model is well suited to study positively selected rare genetic events. aCGH confirmed the precision with which excision at the expected loci is accomplished and ruled out unwanted loss of additional chromosomal material.

Other elegant means of loxP-targeted gRNA delivery to the pancreas such as lentiviral-based methods (44) exist and have been used to edit individual genes. In contrast to the transient expression of CRISPR/Cas9 from the CRE locus, lentiviral injection results in the recombination that results in the loss of insertional mutagenesis, which may confound the phenotypic analysis. Focal delivery of transiently expressed Cas9 and sgRNAs by pancreas electroporation abrogates the risk of insertional mutagenesis, but efficiently transfects only a few hundred cells (67,68). The presence of only a few genetically modified cells is likely not sufficient for the robust phenotypic manifestations of particularly rare events, including homologous fusions.

In summary, the strength of the OMM-ESC approach in combination with the CRISPR/Cas9 technology lies in the genetic flexibility of the system, the temporal control of organ-specific genetic alterations, as well as in the continuous presence of numerously genetically modified cells, thus enabling the rapid phenotypic and functional characterization of positively selected rare genetic events.

Supplementary material
Supplementary data are available at Carcinogenesis online.

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Article

Potent antitumor activity of liposomal irinotecan in an organoid- and CRISPR-Cas9-based murine model of gallbladder cancer

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Abstract: Gallbladder cancer is associated with a dismal prognosis, and accurate in vivo models will be elemental to improve our understanding of this deadly disease and develop better treatment options. We have generated a transplantation based murine model for gallbladder cancer that histologically mimics the human disease, including the development of distant metastasis. Murine gallbladder derived organoids are genetically modified by either retroviral transduction or transfection with CRISPR/Cas9 encoding plasmids, thereby allowing the rapid generation of complex cancer genotypes. We characterize the model in the presence of two of the most frequent oncogenic drivers – Kras and ERBB2, and provide evidence that the tumor histology is highly dependent on the driver oncogene. Further, we demonstrate the utility of the model for the preclinical assessment of novel therapeutic approaches by showing that liposomal Irinotecan (Nal-IRI) is retained in tumor cells and significantly prolongs the survival of gallbladder cancer bearing mice compared to conventional irinotecan.

Keywords: Organoids, gallbladder, CRISPR/Cas9, Nal-IRI, mouse model

1. Introduction

Gallbladder cancer (GBC) is the most common biliary tract cancer, and ranks sixth of all gastrointestinal cancers. In 2018, GBC is predicted to reach more than 200,000 new cases with 165,087 cancer-related deaths worldwide [1,2]. Notably, significant differences in GBC incidence are reported among different geographical regions and ethnicities, with highest rates in South America [3]. These differences may in part be attributed to the prevalence of known risk factors that predispose to the development of GBC, such as the presence of gallstones, chronic bacterial infection (e.g. salmonella), or anomalies of the pancreatobiliary duct junction [4,5]. The median survival of GBC worldwide is low, ranging from 5.7 months to 12.89 months [6,7]. Surgical resection can improve the five year survival
rate, but less than 40% of patients are amenable for surgical resection [8]. Based on the results from the ABC-02 trial published in 2010, combination chemotherapy with gemcitabine and cisplatin remains the standard of care for the treatment of patients with cancers of the biliary tract, including GBC, leading to a median overall survival of 11.7 months with a median progression free survival of 8 months [9]. No established second line concepts exist, but, recently presented results from the first prospective randomized phase III trial, (ABC-06) provide initial evidence that patients with tumor progression under first-line CT can benefit from FOLFOX chemotherapy in the second line setting [10]. However, to date no therapeutic regimen has achieved long-term disease control in GBC. Among others, a novel liposomal formulation of irinotecan (Nal-IRI), which has demonstrated superior performance in patients with advanced pancreatic cancer in combination with leucovorin and 5-fluorouracil [11], is currently being assessed in clinical studies in combination with 5-FU for first and second line treatment of biliary tract cancers, including GBC (1st line: NIFE [12], 2nd line NaliriCC [13]). Irinotecan, one of the most prevalent topoisomerase inhibitors, is a prodrug also known as CPT-11, that undergoes enzymatic activation to its active metabolite SN-38 through the action of carboxylesterases [14]. The liposomal formulation of irinotecan changes the pharmacologic characteristics of irinotecan and has been shown to have superior anti-tumor activity compared to conventional irinotecan in mouse xenograft models [15,16].

Apart from “classical” chemotherapeutic regimens, the potential value of precision oncology is increasingly recognized. Thus far, several oncogenic driver mutations have been identified in patients with gallbladder cancer, including frequent inactivating mutations in tumor suppressor genes like TP53, ARID1A and SMAD4, as well as activating mutations in the KRAS gene. Recurrent amplifications or activating mutations in members of the ERBB2 pathway (EGFR, ERBB2, ERBB3 and ERBB4 and their downstream targets) point towards a decisive role of this pathway in gallbladder carcinogenesis [17-20]. Overall, the molecular landscape of gallbladder carcinoma is heterogeneous, and the consequences of specific genetic aberrations alone or in the context of the co-mutational spectrum remains largely elusive.

In order to functionally annotate the mutational landscape of GBC and to facilitate meaningful pre-and co-clinical trials, genetically flexible in vivo models mimicking the human disease are urgently needed. Immunocompetent in vivo systems serve as a preclinical platform to assess the therapeutic efficacy and characterize the pharmacodynamic properties of novel systemic therapeutic approaches within a complex environment. An existing traditional transgenic mouse models for GBC relies on gallbladder directed overexpression of rat ERBB2. While this model recapitulates several relevant histological features of human GBC, the integration of additional alleles or other driver oncogenes requires time consuming breeding of mice [21].

In this study, we use murine gallbladder organoids to generate a genetically flexible model that allows the study of gallbladder carcinogenesis in the presence of an intact immune system. We show that expression of mutant Kras or mutant ERBB2 (ERBB2^{S310F} and ERBB2^{V777L}), two of the most frequent oncogenic drivers in human GBCs, drives rapid tumor development in vivo in the presence of p53 loss. Further, we demonstrate how the model can be used to functionally validate candidate tumor suppressor genes using CRISPR/Cas9. Importantly, resulting tumors histologically resemble their human counterparts and lead to metastatic spread upon orthotopic transplantation. In order to demonstrate the utility of the model to elucidate relevant pharmacodynamic properties of novel drugs, we show that GBC bearing mice treated with Nal-IRI survive longer than mice receiving conventional irinotecan and that this effect correlates with the prolonged presence of the compound in the epithelial tumor cell compartment.

2. Results

2.1. Introduction of cancer drivers into GB organoids leads to tumor formation in mice

To assess whether gallbladder organoids can be used to study gallbladder carcinogenesis in vivo, we isolated organoids from whole murine gallbladders (Figure 1A-C). As expected, these cells express markers of biliary differentiation, such CK19, Sox9 and EpCAM (Figure 1D,E). Considering that
EpCAM is uniformly expressed by the epithelial cells lining the luminal site of the gallbladder, it appears likely that the cell of origin of gallbladder organoids resides within this compartment (Figure 1B).

TP53 and KRAS are among the most frequently mutated genes in GBC [7,18]. To investigate whether alteration of these genes in gallbladder organoids leads to GBC, we first generated organoids from Kras<sup>G12D</sup> mice. Activation of the latent Kras mutant and loss of p53 with and without loss of Pten was achieved by co-transfecting p3-PGK-Blasticidin-P2A-EGFP and a plasmid co-encoding Cre recombinase, Cas9 and either a single sgRNA against p53, or two sgRNAs targeting p53 and Pten (Fig 2A) [22], followed by selection with blasticidin. An sgRNA directed against a non-genic region on chromosome 8 (sgCR8) [23] served as a negative control. Efficient genome editing was confirmed after selection and expansion by T7 endonuclease assays (Figure 2B).

**Figure 1: Gallbladder organoids express a biliary marker profile**

(A) Technical outline: organoids were isolated from the gallbladders of adult mice, expanded in Matrigel, and genetically modified using CRISPR/Cas9 or by retroviral introduction of cDNAs. Genetically altered organoids were transplanted into recipient mice, either s.c. or orthotopically into the gallbladder. (B) IHC confirms EpCAM expression within the epithelial layer of adult murine gallbladders. (C) Brightfield image of gallbladder organoids. (D) Flow cytometry analysis for EpCAM on single cell suspensions from adult mouse liver (left column), adult mouse gallbladder (middle column) and gallbladder organoids (right column). (E) Immunofluorescence on gallbladder organoids confirms expression of β-catenin (left), CK19 (middle) and SOX9 (right).

Following transplantation, we observed tumor growth in the Kras<sup>G12D/wt;sgp53</sup> (KP) and Kras<sup>G12D/wt;sgp53;sgPten</sup> (KPP) cohorts, but not in animals injected with Kras<sup>G12D/wt;sgCR8</sup> organoids.
Manuscript III: Potent antitumor activity of liposomal irinotecan in an organoid- and CRISPR-Cas9-based murine model of gallbladder cancer, Accepted 26.11.2019, Cancers (Post-Print)

(KCR8) (Figure 2C). Accordingly, recipient mice reached endpoint criteria with a median latency of 46 days vs. 69 days after implantation of organoids in the KPP and the KP cohort, respectively (Figure 2D).

Histological examination of the tumors in both the KP and KPP cohorts revealed mostly tubular adenocarcinomas with areas of mucin production, as assessed by Alcian blue staining (Figure 2E, Suppl. Table 1). Loss of PTEN in the tumor cells, but not in the recipient-derived stromal cells was confirmed by IHC on histological sections (Figure 2E). Loss of PTEN in KPP and loss of p53 in KP and KPP tumor derived cell lines was detected by western blot (Figure 2F, Figure S1). The increased frequency of indels in tumor derived cell lines compared to preinjection organoids indicates positive selection of the targeted genes p53 and Pten during tumor development (Figure 2G). As expected, the majority of indels are predicted to cause frameshifts (for details see Figure S2). Since a prominent stromal reaction is a hallmark of GBC, we quantified the CK19 negative area as a surrogate for the relative contribution of the tumor stroma to the tumor volume. Approximately 57% of the tumors stained CK19 negative, with no significant differences between the KP and KPP groups (57.44% vs 56.97%) (Figure 2H). Thus, tumor development in the KP and KPP cohorts validates the suitability of our model to generate GBCs with complex cancer genotypes in vivo using CRISPR/Cas9 that histologically resemble crucial characteristics of the human disease.

Figure 2: Genetically modified gallbladder organoids can give rise to GBC that resembles the human disease

(A) Schematic of plasmids used to transfect gallbladder organoids. Plasmids contain Cre recombinase, Cas9 and the respective sgRNA(s). (B) T7 endonuclease assay confirming cleavage after transfection and selection with blasticidin, first column: KCR8 organoids, second column: KP organoids and

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third column: KPP organoids; arrows indicate cleaved bands. (C) Tumor volume 32 days after organoid implantation. No tumor development occurred in mice transplanted with KCR8 organoids during the 4 months observation period. (D) Kaplan-Meyer curves of mice transplanted with KCR8, KP, and KPP organoids. Transplantation with KP and KPP organoids led to rapid tumor development (median survival: 69 days and 46 days for the KP and KPP cohorts, respectively). (E) Histological characteristics of GBC tumors derived from KP and KPP organoids. H&E staining of both genotypes shows GBCs classified as adenocarcinomas. IHC for CK19 confirms ductal differentiation and PTEN IHC detects loss of PTEN expression in sgPten-bearing epithelial tumor cells, but not in the surrounding stroma cells. IHC for αSMA confirms the presence of cancer-associated fibroblasts. Normal gallbladder tissue (H&E) for comparison. (F) Loss of p53 and PTEN confirmed on tumor-derived cell lines of the respective genotypes by immunoblotting. KCR8 organoids served as positive control. (G) Frequency of indels in the respective loci in preinjection organoids and in tumor derived cell lines shows enrichment of p53- and PTEN alterations during tumor development. (H) The relative stromal content of KP and KPP derived tumors (considering CK19 negative area as a surrogate for the relative stromal content) did not differ significantly (57.44% and 56.97%, respectively; P > 0.8669).

2.2. Tumors derived from orthotopic transplantation of genetically altered organoids frequently metastasize to the lung

Next, we assessed whether the histological presentation and/or the development of metastatic spread in our murine GBC model depends on the site of implantation. KPP organoids were either injected orthotopically into the gallbladder or subcutaneously (s.c.) into the flanks of recipient mice. Histologically, tumors from both sites presented as adenocarcinomas, with a moderately increased stromal content in the orthotopic group as assessed by CK19 negative area (Figure 3A,B). Notably, lung metastatic disease was exclusively detected in 50% of orthotopically transplanted mice, but in none of the mice that received flank injections (Figure 3C). Compared to the parental tumors, the metastases within the lung displayed dense aggregates of tumor cells and a significantly reduced stromal content compared to the parental tumor as assessed by CK19 negative area (Figure 3D,E).
Figure 3: Genetically altered gallbladder organoids lead to metastasis upon orthotopic implantation

(A) Both s.c. and orthotopically implanted KPP organoids lead to GBCs classified as adenocarcinomas. (B) Orthotopic GBCs presented with a larger stromal compartment in as assessed by quantification of the CK19 negative area (54.15% and 62.69%, respectively (n = 9 & n = 7, respectively, P=0.0421)). (C) Lung metastases were present in 5/10 mice after orthotopic transplantation and in 0/10 mice after s.c. transplantation with KPP organoids. (D) H&E and CK19 staining of lung metastasis. (E) Compared to the parental orthotopic tumors, CK19 positive area as a surrogate for relative stromal content is significantly reduced in lung metastases (62.69% and 38.48% respectively, n = 7 & n = 3, respectively, P=0.0026).

2.3. Overexpression of activating ERBB2 mutants give rise to GBC

Mutations in the ERBB2-gene are among the most common genetic alterations in gallbladder cancer [17,18,24-26]. To assess their potential as oncogenic drivers in our organoid based GBCs, we stably introduced human ERBB2 and two ERBB2 mutants (ERBB2^{S310F} and ERBB2^{V777L}) by retroviral transduction into gallbladder organoids, in which p53 loss had been induced by Cas9-mediated genome editing (Figure 4A). Membranous expression of both wild-type (WT) ERBB2 and both ERBB2 mutants as well as the respective phosphorylated proteins on transduced gallbladder organoids was confirmed by immunofluorescence (Figure 4B).
Figure 4: Mutant ERBB2 cooperates with loss of p53 and leads to papillary GBC in recipient mice

(A) Top: Schematic of human ERBB2, indicating the location of two point mutants (S310F and V777L). Bottom: retroviral vector used to transduce organoids, that had been treated with an sgp53-containing plasmid (px459) to induce loss of p53. (B) Immunofluorescence for ERBB2 (top) and phospho-ERBB2 (bottom) on organoids harboring the indicated genetic alterations. (C) Tumor volumes 36 days after s.c. implantation of the respective organoids into recipient mice. All mice transplanted with sgp53;ERBB2\textsuperscript{S310F} and sgp53;ERBB2\textsuperscript{V777L} organoids exhibited tumor development, whereas sgp53;empty vector- and sgp53;ERBB2\textsuperscript{wildtype} organoids did not give rise to tumors over a 4 months observation period. There was no significant difference in the tumor burden of mice transplanted with sgp53;ERBB2\textsuperscript{S310F} and sgp53;ERBB2\textsuperscript{V777L} organoids (P= 0.999). (D) Mice transplanted with sgp53;ERBB2\textsuperscript{S310F} and sgp53;ERBB2\textsuperscript{V777L} organoids reached endpoint criteria with a median survival of 79.5 days and 58.5 days, respectively. (E) H&E and IHC for CK19 and EGFP on tumors generated with sgp53;ERBB2\textsuperscript{S310F} and sgp53;ERBB2\textsuperscript{V777L} organoids.

Both ERBB2 mutants cooperated with p53 loss and gave rise to GBC with a median OS of 79.5 and 58.5 days for ERBB2\textsuperscript{S310F} and ERBB2\textsuperscript{V777L}, respectively, whereas wildtype ERBB2 unexpectedly did not lead to tumor development within the observational period of 4 months (Figure 4C,D). Compared to GBCs harboring Kras\textsuperscript{G12D} (KP and KPP), mutant ERBB2-driven tumors displayed distinct histological characteristics: while nearly all mutant Kras-driven GBC were classified as tubular adenocarcinomas, mutant ERBB2-driven GBCs were mostly of papillary/tubulo-papillary differentiation (Figure 4E, Suppl. Table 1). Both genotypes led to stromal desmoplasia (Figure 2E, Figure 4E).

In summary, we show that the model histologically recapitulates prime hallmarks of the human disease and that its histology is dependent on the driving oncogenes.

\textbf{2.4. Antitumor effects of Nal-IRI correlate with increased intratumoral CPT-11 concentrations}

The topoisomerase inhibitor Nal-IRI achieved a significant increase in median overall survival of previously treated patients with pancreatic cancer [11]. Since pancreatic cancer shares several features of biliary tract cancers, such as the abundant stromal desmoplasia and the relative chemotherapy resistance, we wanted to assess whether Nal-IRI leads to a survival benefit in our stroma-rich GBC model in comparison to conventional irinotecan. First, we tested whether Carboxylesterase 2 (CES2), the enzyme, that catalyzes the activation of irinotecan (CPT-11) to the active compound SN-38, is expressed in our murine organoid derived GBCs. IHC confirmed that CES2 is expressed in tumor cells as well as in the stromal cell compartment, suggesting that both cellular compartments are capable of activating CPT-11 to SN-38 (Figure 5A). We transplanted KPP organoids into recipient mice and, when tumor sizes reached 150 mm\textsuperscript{3}, animals were randomized into four treatment arms (vehicle, irinotecan 50 mg/kg, Nal-IRI 25 mg/kg and Nal-IRI 50 mg/kg). Nal-IRI administered at 50 mg/kg lead to significant reduction in tumor size and prolonged the median survival to 33 days compared to 22 days for vehicle-treated mice, 21 days for free irinotecan, and 25 days for Nal-IRI administered at 25 mg/kg (Figure 5B,C). This is in stark contrast to the in vitro situation, in which Nal-IRI exhibits higher IC50s compared to free Irinotecan (Figure 5D).
Figure 5: Treatment with Nal-IRI leads to improved survival in GBC bearing mice

(A) IHC for CES2 on a tumor derived from KPP organoids confirms CES2 expression in both the tumor cell compartment and in stromal cells. (B) Waterfall plots displaying growth fold changes after 16 days of vehicle, irinotecan, Nal-IRI 25mg/kg or Nal-IRI 50mg/kg treatment. Mean fold changes are 5.87, 5.14, 3.38 and 2.60, respectively. Treatment with Nal-IRI 50mg/Kg led to significantly reduced tumor growth compared to vehicle (P=0.0232). (C) Kaplan-Meyer curve of vehicle, irinotecan, Nal-IRI 25 mg/kg or Nal-IRI 50mg/kg treated GBC-bearing mice. Median survivals were 22 days, 21 days, 25 days and 33 days, respectively. Nal-IRI 50mg/kg led to a significantly improved survival compared to vehicle and free irinotecan (P= 0.0015 and P=0.0047, respectively). (D) IC50s of a KPP GBC-tumor derived cell line for irinotecan and Nal-IRI in vitro. (E) Intratumoral CPT-11 concentration of vehicle-, irinotecan- or Nal-IRI- treated, tumor-bearing mice 72 hours after a single injection with the respective drug. Treatment with Nal-IRI led to higher intratumoral CPT-11 levels than treatment with free irinotecan (P=0.0006). (F) Intratumoral SN-38 concentrations 72 hours after a single injection with vehicle-, irinotecan- or Nal-IRI. SN-38 is exclusively detectable in Nal-IRI treated mice (P=0.0006). (G) CPT-11 concentrations within tumor- and stromal cells from vehicle, irinotecan or Nal-IRI treated tumor-bearing mice, 72 hours after a single injection. EGFP-labelled tumor cells were separated from the EGFP-negative stromal cells by FACS and subsequently analyzed with LC-MS/MS. Mean CPT-11 concentrations were significantly higher in tumor cells than in stromal cells (P=0.0001) in Nal-IRI treated mice.

Next, we aimed to delineate whether administration of Nal-IRI leads to higher intratumoral concentrations of CPT-11 and its active metabolite, SN38, compared to free irinotecan and reaches the tumor cell compartment despite the abundant desmoplasia in mice bearing s.c. GBCs. Tumors were harvested 72 hours after a single treatment with vehicle, Nal-IRI (50 mg/kg), or irinotecan (50 mg/kg). Using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), we quantified the levels of CPT-11 and SN-38. Three days after injection, both CPT-11 (Figure 5E) and SN-38 (Figure 5F) were significantly higher in lysates from the Nal-IRI treated GBCs than in tumor lysates from mice treated with free irinotecan.
The tumor stroma may serve as a barrier for efficient drug delivery to tumor cells and stromal cells have the potential to scavenge cytostatic drugs, thereby affecting the pharmacokinetics and pharmacodynamics of drugs [27,28]. Considering that the previous experiment was performed on whole tumor lysates, we aimed to address whether Nal-IRI is predominantly retained within the tumor cells or the stroma cell compartment. To do so, we derived tumors from KPP organoids stably transfectd with an EGFP expression cassette. 72 hours following a single injection of vehicle, Nal-IRI (50 mg/kg), or irinotecan (50 mg/kg), EGFP positive tumor cells and EGFP negative stromal cells were separated by FACS, and the individual fractions were subjected to LC-MS/MS analysis. Confirming our previous results from whole tumor lysates, we detected more abundant CPT-11 in the Nal-IRI treated mice than in mice receiving conventional irinotecan in both the stromal cells and the tumor cells. Furthermore we found significantly higher CPT-11 levels in the tumor cells as compared to the EGFP negative stromal cells (Figure 5G).

Together, the improved survival of GBC bearing mice treated with Nal-IRI over conventional irinotecan is paralleled by a prolonged presence of the active drug within the tumors, where it is predominantly retained in tumor cells and not in stromal cells. These data also illustrate that our model is particularly well suited for pharmacologic investigations due to its intact microenvironment resembling the human disease.

3. Discussion

Recurrent key genetic alterations in patients with GBC lead to inactivation of the tumor suppressor TP53 (47.1%), to oncogenic activation of KRAS (7.8%), or to increased signaling through various components of the ERBB pathway (36.8%). In addition, multiple other genes, such as RNF43, FBXW7, MAP2K4, have been found to be mutated, albeit at considerably lower frequency [17].

A murine model for GBC (BK5.ERBB2 mice) exists but relies on time consuming traditional breeding [29]. Considering that cancer therapy is increasingly moving towards personalized approaches, genetically flexible model systems are needed to adequately model also more complex genetic phenotypes found in GBC patients. Here, we present a murine model for GBC that relies on key tumorigenic drivers, but can be easily adapted in an individualized fashion to assess the potential influence of the co-mutational spectrum on tumorigenesis and therapy response.

Organoid cultures have been established from various murine and human tissues. These cultures allow for the propagation of both normal and malignant cells and have opened up new avenues for cancer research including screens for novel therapeutics (reviewed in [30]). Murine gallbladder organoids can be passaged for long periods of time, are able to undergo repeated freeze/thaw cycles and can be transplanted into syngeneic recipient mice. Using untransformed murine organoids instead of fully transformed human tumor cell lines not only allows researchers to study carcinogenesis starting from a wildtype cell, but also enables them to investigate GBC development and treatment strategies in the presence of an intact immune system.

We use murine gallbladder derived organoids to demonstrate how activation of mutant Kras or ERBB2 in conjunction with loss-of-function of single or multiple tumor suppressor genes reliably leads to GBC in recipient mice. Disruption of candidate tumor suppressor genes or activation of latent alleles is efficiently accomplished by transfection of CRISPR-Cas9-encoding plasmids or Cre-recombinase, respectively, while retroviral transduction facilitates the rapid introduction of cDNAs encoding wildtype or mutant proteins. We generate ortho- and heterotopic GBCs featuring the most frequent genetic alterations (p53 together with mutant KRAS, as well as p53 in conjunction with mutant ERBB2). Tumors develop with 100% penetrance and can be generated with and without loss of Pten, a gene that is inactivated in a subset of human GBCs [18,31]. Since murine Erbb2 is known to be less oncogenic than its human counterpart, we introduced the human ERBB2 gene [32]. Interestingly, while ERBB2 is frequently amplified in various malignancies, including breast cancer, gastric cancer, or colon cancer [33-35], GBCs have a substantial rate of ERBB2 mutations [17,18,24]. The ERBB2 mutants ERBB2S310F and ERBB2V777L used in this work are located in the extracellular domain and in the tyrosine kinase domain, respectively, and lead to enhanced downstream signaling [36,37]. Notably, in our model, tumor development only occurred in the presence of the ERBB2 mutants, but not upon overexpression of the
WT human ERBB2, further substantiating the notion that mutant ERBB2 is a more potent cancer driver than overexpression of WT ERBB2. This data is in line with results from experiments in breast cancer (reviewed in [38]).

Histologically, both mutant KRAS and mutant ERBB2 driven tumors resemble human GBCs. Interestingly, the mutant KRAS-driven GBCs predominantly led to adenocarcinomas with tubular structures whereas the ERBB2-driven GBC frequently showed a pronounced tubulo-papillary/papillary differentiation. Both genotypes led to stromal desmoplasia, a hallmark of GBC and an important feature since the influence of the stromal compartment on therapy resistance is increasingly recognized in pancreato-biliary cancers [39-42]. Murine models that accurately depict the histology and microenvironment of human tumors are particularly important to create an adequate preclinical in vivo situation for the testing of novel therapeutic compounds: despite exhibiting a potent anti-tumor activity either in vitro or in tumors derived from the implantation of tumor cell lines, several chemotherapeutic compounds failed in the clinical setting [43,44]. This may, in part, result from the lack of the complex interactions of a drug with the multiple different cell types and extracellular matrices present within the tumor microenvironment.

Irinotecan is a topoisomerase inhibitor that is frequently used in combination with fluorouracil (5-FU) based chemotherapeutic regimens. It is processed to its active metabolite SN-38 by CES-enzymes, and prompted for inactivation through the conversion to a glucuronide derivate (SN-38G) as the main excreted metabolite by UDP-glucuronyltransferases [14]. Since the activity of irinotecan is limited due to major side effects and a short half-life, liposomal delivery systems have been developed. The anti-tumoral activity of Nal-IRI has been found exceed that of free irinotecan in a mouse xenograft model of colon cancer [45]; and in human patients with metastatic pancreatic cancer, Nal-IRI achieved superior overall survival in combination with fluorouracil and leucovorin [11]. Stromal desmoplasia in pancreatic cancer can act as a barrier to chemotherapeutic agents [28]. Therefore, we wanted to delineate whether Nal-IRI is capable of sufficiently penetrating the GBC stroma and to reach relevant CPT-11 and SN-38 levels within the tumor cell compartment. Since both tumors cells and stromal cells express CES2, both compartments are likely capable of generating the active metabolite SN-38.

Although the in vitro activity of Nal-IRI was lower than for free irinotecan, weekly administration of Nal-IRI 50 mg/kg lead to a significant survival benefit of tumor-bearing mice, while free irinotecan was not superior to vehicle in our model. The survival benefit may in part be due to the extended levels of CPT-11 within our GBCs, as we detected substantially higher levels of both CPT-11 and SN-38 in tumors of Nal-IRI treated mice 72 hours after treatment. However, we also show a relative enrichment of CPT-11 in tumor cells over stromal cells, indicating that Nal-IRI accumulates preferentially within GBC cells and suggesting that a liposomal formulation may be beneficial in stroma-rich tumors.

4. Materials and Methods

4.1. Animal experiments

Mice were maintained under standard housing conditions with access to food and water ad libitum and a 12-hour day-night cycle. All interventions were conducted during the day cycle. Kras\textsuperscript{lt;G12D} mice [46] were a gift from Dieter Saur (Munich, Germany). Recipient mice (C57BL/6) and NOD.Cg-Prkd\textsuperscript{enu}K12D\textsuperscript{wtfrtj/hsz} (NSG, 5-8 weeks old) were purchased from the local animal facility (Hannover Medical School, Germany). C57BL/6 mice were used as recipients for organoids derived from C57BL/6 or syngeneic Kras\textsuperscript{lt;G12D} mice. NSG mice were used as recipients for organoids transduced with human ERBB2 proteins. Mouse experiments were approved by local authorities (the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES)). Mice were harvested when they reached endpoint criteria (sign of ill health, tumor volume > 1200 mm\textsuperscript{3}).

4.2. Isolation of murine gallbladder organoids

Murine gallbladder organoids were isolated from adult C57BL/6 mice or Kras\textsuperscript{lt;G12D} mice with some modifications to published protocols [47]. Briefly, the murine gallbladder was minced with a scalpel
and filtered through a 100 µm mesh. After additional washes with PBS, cells were spun at 300g for 5 minutes, resuspended in 100% Growth Factor Reduced Matrigel (Corning, NY), and plated in a 24-well plate (two 50 µL droplets per well). After solidification, Matrigel droplets were overlaid with 500 µL murine liver organoid media according to published protocols [47]. For passaging, organoids were mechanically disrupted by repeated pipetting using a P200 pipette tip, followed by a 3- to 5-minute enzymatic digestion in TrypLE Express solution (Thermo Fisher, Waltham, MA).

4.3. Tumor Cell Isolation

Organoid derived tumors were minced with a scalpel and enzymatically digested in a shaking incubator with Collagenase IV 1 mg/mL (Sigma-Aldrich, St Louis, MO) in EBSS (Thermo Fisher, Waltham, MA) for 1 hour at 37°C. Cells were washed with PBS, spun at 300g, resuspended and plated on tissue culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

4.4. IC50 Cell Viability Assay:

For inhibitor treatment, tumor cell lines established as 2D cultures from primary organoid-derived tumors were plated at 5,000 cells per 96-well and treated with irinotecan-HCl (Aurobindo Pharma, Munich, Germany) or Nal-IRI (Onyvide, Servier, Neuilly-sur-Seine, France) for 72 hours. At the indicated time points, luminescence was assessed using the CellTiter-Glo Luminescent Cell Viability Assay on a Glomax Multi Detection System (Promega, Madison, WI).

4.5. Flow Cytometry and Cell Sorting

Single cell suspensions from murine gallbladder organoids were prepared and incubated with the primary antibody (1:100 dilution) for 30 minutes at 4°C (Allophycocyanin-EpCAM, ThermoFisher Scientific, Waltham, MA, Cat. #17-5791-80). Flow cytometry was performed on a FacsCanto (BD Bioscience, San Jose, CA) and analysis was performed using Flowjo (Flowjo LCC, Oregon, USA). For cell sorting, single cell suspensions were prepared from organoid derived tumors as described above. EGFP-positive and –negative cells were separated by fluorescence activated cells sorting (FACS) at the institutional cell sorting facility (Hannover Medical School, Germany).

4.6. Subcutaneous and orthotopic transplantation of organoids

For subcutaneous (s.c.) injections, 0.5 × 10^5 organoids were resuspended in 50 µL DMEM F12/Advanced with 50% Growth Factor Reduced Matrigel (Corning, NY) and injected s.c. into the rear flanks of recipient mice. For orthotopic transplantation, mice were starved for 2 hours before the surgery. A subternal 5 mm longitudinal incision was performed, the gallbladder was exposed, and the bile was aspirated using a 31G syringe (BD Medical, France #324826). Subsequently, 0.5 × 10^5 organoids were resuspended in 10 µL of 100% Growth Factor Reduced Matrigel (Corning, NY) and were implanted using a 31G syringe (BD Medical, France #324826). After retraction of the needle, the injection site was compressed with a sterile cotton swab, and the abdominal cavity was washed with 2 mL of sterile pre-warmed water. The abdominal wall was closed layer-wise using absorbable sutures.

4.7. Plasmids

The U6-sgRNA-Cas9-P2A-Cre plasmid was a gift from Lukas E. Dow. sgRNA against Cr8, p53, and Pten were inserted as described previously [48]. The pMSCV-ERBB2-IRES-EGFP was a gift from Martine Roussel (Addgene, plasmid #91888). ERBB2 mutants were generated via site-directed mutagenesis PCR. The sgRNA against p53 was cloned into pX459 as described previously (Addgene, plasmid #48139) [49].

4.8. Transfection and retroviral transduction of organoids

Gallbladder organoids derived from Kras^{hG12D} mice were transiently cotransfected with pt3-PGK-Blasticidin-P2A-EGFP and either U6-sgCr8-EFS-Cas9-P2A-Cre (KCR8 organoids), U6-sgp53-EFS-Cas9-
P2A-Cre (KP organoids) or U6-sgp53-U6-sgPten-EFS-Cas9-P2A-Cre (KPP organoids) using Lipofectamine2000 (ThermoFisher Scientific, Waltham, MA) and selected with blasticidin (20 µg/mL). Prior to transduction with different ERBB2 expressing retroviruses we transfected gallbladder organoids from C57BL/6j mice with px459_sgp53 and selected with puromycin (50 µg/mL).

To mark organoids with a green fluorescent marker (EGFP), we cotransfected pt3-PGK-Blasticidin-P2A-EGFP with the sleeping beauty-13 plasmid (kindly provided by David A. Largaespada, University of Minnesota) using Lipofectamine2000 and selected with blasticidin (20 µg/mL). MSCV-based retroviruses (pMSCV-ERBB2-IRES-EGFP) were produced in Platinum-E retroviral packaging cells (Cell Biolabs, San Diego, CA), concentrated using Retro-X concentrator (Clontech, Mountain View, CA), and supplemented with polybrene (4 µg/mL) prior to transduction of organoids.

4.9. T7-Endonuclease assays and quantification of indel frequency in edited organoids and tumor derived cell lines

Cas9-mediated DNA cleavage with sgCr8, sgP53 and sgPten were verified using the T7 Endonuclease I EnGen Mutation Detection Kit (NEB, Ipswich, MA) according to the manufacturer’s manual. PCR products were heteroduplex annealed and treated with Endonuclease T7. Next generation sequencing to determine indel frequency was performed at the genomics core unit at Hannover Medical School. Target regions from genomic DNA were amplified using corresponding primers, the PCR amplicons were pooled per sample in equimolar concentrations. The sequencing fragment libraries were prepared from 50 ng DNA with the NebNext Ultra II DNA Library Prep Kit from NEB, following the manufacture’s protocols. Sequencing was performed on a MiSeq (Illumina) Nano Flowcell. Indel frequency was determined by filtering the fastq reads for the target region and the 20bp sequence surrounding the expected cleavage site of the respective sgRNA and direct counting of WT and indel reads. The analysis of editing events was performed using the ampliCan ([50]) method. We created a design table with our amplicon sequences, primers and sgRNA sequences for all five samples, together with the raw MiSeq sequencing data. With the aforementioned MiSeq data and the created design table we employed the ampliCanPipeline method to compute summary metrics such as the number of frameshifts, and several other metrics. The analysis was performed using GNU R using a Jupyter Notebook ([51]). Downstream analysis plots for the size of observed indels were plotted using ggplot2’s violin-plot functions to describe the variance in indel sizes on a sample level appropriately. All other final results were plotted using ggplot2 ([52]) in R using a custom analysis script.

Guide RNA sequences:

<table>
<thead>
<tr>
<th>p53 sgRNA</th>
<th>CCTCGAGCTCCCTCTGAGCC</th>
</tr>
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<tbody>
<tr>
<td>Pten sgRNA</td>
<td>GAGATCGTTAGCAGAACAATAA</td>
</tr>
<tr>
<td>Cr8 sgRNA</td>
<td>GACATTCTTTCCCTCCTGGA</td>
</tr>
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</table>

81
### Primers used in T7 Endonuclease Mutation Detection Assay:

<p>| | |</p>
<table>
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<th></th>
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<td>T7 Mut PCR p53 fwd</td>
<td>GCCATCTTGGGTCTTGACTT</td>
</tr>
<tr>
<td>T7 Mut PCR p53 rev</td>
<td>CCCGCAGGATTACAGACA</td>
</tr>
<tr>
<td>T7 Mut PCR <em>Pten</em> fwd</td>
<td>GAGCCATTCCATCTGCAG</td>
</tr>
<tr>
<td>T7 Mut PCR <em>Pten</em> rev</td>
<td>CTAGCCGAACACTCCCTAGG</td>
</tr>
<tr>
<td>T7 Mut PCR Cr8 fwd</td>
<td>TAAGATGATTATCGAATCCG</td>
</tr>
<tr>
<td>T7 Mut PCR Cr8 rev</td>
<td>TCTTATCCCCTGTGTTGGAA</td>
</tr>
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</table>

### Primers used in NGS:

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<th></th>
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<tr>
<td>NGS PCR p53 fwd</td>
<td>CCATAGGAGTGTGTGTTGTTG</td>
</tr>
<tr>
<td>NGS PCR p53 rev</td>
<td>CGCAGGATTACAGACACCC</td>
</tr>
<tr>
<td>NGS PCR <em>Pten</em> fwd</td>
<td>GAGCCATTCCATCTGCAG</td>
</tr>
<tr>
<td>NGS PCR <em>Pten</em> rev</td>
<td>CAGATCTAGAAATGCGCCC</td>
</tr>
<tr>
<td>NGS PCR Cr8 fwd</td>
<td>TCTGAATTCTGGGATGGGG</td>
</tr>
<tr>
<td>NGS PCR Cr8 rev</td>
<td>TGTGTGGCTACCTGTCTT</td>
</tr>
</tbody>
</table>

### 4.10. Immunohistochemistry, Immunofluorescence and Alcian Blue:

Paraffin-embedded tissue slides were deparaffinized and rehydrated. For Alcian-Blue staining (Serva Electrophoresis, Heidelberg, Germany) the deparaffinized slides were immersed in 3% acetic acid and stained in Fast Red for 30 seconds. Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) were performed as described [53]. For IHC, we used the following primary antibodies: Abcam (Cambridge, UK): CK19 (ab133496), αSMA (5694) were diluted 1:250; Cell Signaling Technology (Danvers, MA): PTEN XP (9188s) was diluted 1:200; Santa Cruz (Dallas, USA): Ep-CAM (sc-66020) was diluted 1:200; Merck (Darmstadt, Germany): CES2 (ABS1065) was diluted 1:200. The secondary biotin-conjugated antibody (goat-anti-rabbit, #B-2770, Life Technologies, Carlsbad, CA) was diluted 1:250. For immunofluorescence, Santa Cruz Biotechnology: β-Catenin (sc-7963), CK19 (sc-33111), and Sox9 (sc-20095); Cell Signaling Technology (Danvers, MA): ERBB2, (2165s); Novus Biological (Colorado, USA): p-ERBB2 (NB100-81960) were all diluted 1:50.
4.11. Immunoblotting

Immunoblotting was performed as previously described [53]. We used the following primary antibodies: Cell Signaling Technology (Danvers, MA): PTEN XP (9188S), ERBB2 (21655), were diluted 1:1000, Vinculin XP (13901) was diluted 1:5000; Novus Biological (Colorado, USA): p-ERBB2 (NB100-81960) was diluted 1:1000; Leica Biosystem (Illinois, USA): p53 (P53-PROTEIN-CM5) 1:1000. Secondary antibodies: Cell Signaling Technology: goat-anti-rabbit (7074S) was diluted 1:1000. For the p53 western blot organoids and tumor derived cell lines were treated with Doxorubicin (1ng/mL) for 4 hours.

4.12. In vivo chemotherapy treatment

C57Bl/6 mice were injected s.c. with KPP organoids and randomized upon detection of a tumor of 150 mm$^3$ into one of three treatment arms (Irinotecan, n = 6, 50 mg/kg, intravenous, Aurobindo Pharma, Munich, Germany) or (Nal-IRI, n = 6, 25 mg/kg, intravenous, Onyvide, Servier, Neuilly-sur-Seine, France) or (Nal-IRI, n = 6, 50 mg/kg, intravenous, Onyvide, Servier, Neuilly-sur-Seine, France) or vehicle (NaCl 0.9%, n = 6) arm. Tumor growth was followed by caliper measurements and mice were harvested upon reaching endpoint criteria (tumor volume > 1200 mm$^3$, signs of ill health).

To quantify the levels of SN-38 and CPT-11 in GBCs we treated mice harboring a GBC (KPP organoids) of > 500 mm$^3$ with a single treatment of either vehicle, Irinotecan (50 mg/kg) or Nal-IRI (50 mg/kg). 72 hours after the treatment, tumors were harvested and either analyzed directly using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), or FACS-sorted for the EGFP-positive and -negative fractions prior to LC-MS/MS. All LC-MS/MS analyses were done at the metabolomics core facility of Hannover Medical School (MHH, Hannover, Germany).

4.13. Determination of CK19-negative area

The CK19-negative area was determined by automatic thresholding using Fiji, ImageJ (National Institutes of Health, USA). Five non-overlapping low-magnification fields of view were assessed per tumor.

4.14. Statistical analysis

Experimental data were analyzed using GraphPad Prism software. If not stated otherwise, a P-value of < 0.05 was considered significant. We applied a 2-tailed t test to compare the CK19-negative area of tumor samples. We used the Log-rank (Mantel-Cox) test to calculate differences in animal survival. A P-value of < 0.008 was considered significant when making individual comparisons among four different cohorts of tumor bearing mice [54]. 1-way ANOVA with Tukey’s Multiple Comparison Test was used to assess differences in tumor growth and also in CPT-11 and SN-38 levels. 2-way ANOVA with Bonferroni’s test was used to calculate the difference of CPT-11 and SN-38 in different treatments and compartments. Chi-square was used to calculate the proportions of lung metastasis in mice bearing s.c. and orthotopic GBCs.

5. Conclusions

We present and characterize an organoid-based GBC mouse model that facilitates the rapid interrogation of putative cancer genes using CRISPR-Cas9 technology. With its intact tumor microenvironment and the close histological resemblance to human tumors, the model is highly suited to address the efficacy and pharmacodynamic properties of novel therapeutic compounds. We utilize this model to show that Nal-IRI enriches in the tumor cell compartment and prolongs the survival of GBC-bearing mice compared to conventional irinotecan.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Histological classification of organoid GBC tumors, Figure S1: uncropped western blot images, Figure S2: characterization of induced indels.
Author Contributions: Conceptualization, ZE, AS, AV and MS; Data curation, ZE, KW, AP and SN; Formal analysis, ZE, TP, AP, SN and MS; Funding acquisition, AS, AV and MS; Investigation, ZE, KW and SS; Methodology, ZE, KW, AS, AV and MS; Project administration, ZE and MS; Resources, KT, NW, FK, MM, AV and MS; Supervision, MS; Validation, ZE, KW, TP, AP, SN, SS, KT, NW, FK, MM, AS, AV and MS; Visualization, ZE, KW, TF, AP and SN; Writing – original draft, ZE, AS, AV and MS; Writing – review & editing, ZE, KW, TP, SS, KT, NW, FK, MM, AS, AV and MS.

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Conflicts of Interest: Arndt Vogel and Michael Saborowski are recipients of a research grant from Les Laboratoires Servier.

References


12. OncologyPro. Nife-trial: Liposomal irinotecan (nal-iri) plus 5-fluorouracil (5-fu) and leucovorin (lv) or gemcitabine plus cisplatin in advanced biliary-tract c... | oncologypuro.


8 Animal husbandry & experiments

All experimental mice were kept in individually ventilated cages (IVC) with controlled environmental conditions of 21 ±2°C, relative humidity of 55 ±10% and artificial light with a 14:10 light/dark cycle. Autoclaved water and commercial pellet diet (Altromin, Germany) was available ad libitum. Mice were kept in groups up to 5 mice with commercial softwood granulate bedding (Altromin, Germany).

All animal experiments were performed according to protocols approved by the local authority The Lower Saxony State Office for Consumer Protection and Food Safety (LAVES). Mice were routinely monitored for palpable tumors or of sign of ill health and scored according to an activity score modified from van Griensven et al.\textsuperscript{153}, see Table 2.

Table 2: Activity Score according to animal protocols. (Modified Van Griensven et al, Shock 2002)\textsuperscript{153}

<table>
<thead>
<tr>
<th>Score</th>
<th>Erscheinungsbild</th>
<th>Kriterien</th>
<th>Konsequenz</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sehr aktiv</td>
<td>Gesundes Erscheinungsbild, neugierig, rasche, kräftige und adäquate Bewegungen, regelrechte Nahrungsaufnahme, Fell glatt, glänzend</td>
<td>Fortsetzung der routinemäßigen Kontrollen</td>
</tr>
<tr>
<td>2</td>
<td>Aktiv</td>
<td>Gesundes Erscheinungsbild, neugierig, vereinzelte gelegentliche Unterbrechungen der regelrechten Aktivität, regelrechte Nahrungsaufnahme, Fell glatt, glänzend</td>
<td>Fortsetzung der routinemäßigen Kontrollen</td>
</tr>
<tr>
<td>3</td>
<td>Eingeschränkte Aktivität</td>
<td>Adäquate Reaktion auf die Umgebung, häufige Unterbrechungen der Aktivität, leichte Verminderung der Nahrungsaufnahme, Fell stumpf, Augen nicht mehr vollständig geöffnet. Palpabler abdomineller Tumor von 0.5 cm</td>
<td>Tägliche Kontrollen</td>
</tr>
<tr>
<td>4</td>
<td>Verlangsamt</td>
<td>Schläfrig, langsame Bewegungen, deutliche Einschränkung der Nahrungsaufnahme, Fell stumpf, Augen nicht mehr vollständig geöffnet.</td>
<td>Tierschutzgerechtes Töten</td>
</tr>
<tr>
<td>5</td>
<td>Lethargisch</td>
<td>Nur residuelle Aktivität bis bewegungslos, Fell aufgestellt/schmutzig, Augen geschlossen, fehlende Nahrungsaufnahme</td>
<td>Tierschutzgerechtes Töten</td>
</tr>
</tbody>
</table>
8.1 Manuscript I: Murine Liver Organoids as a Genetically Flexible System to Study Liver Cancer In Vivo and In Vitro, Hepatology Communications, 2019

All animal experiments were approved by the LAVES (animal protocols 15/1945 and 17/2553). Mice used for organ removal, subcutaneous or orthotopic injection were over six weeks of age.

8.1.1 Mouse Strains

Table 3: Mouse strains used in Manuscript I, named with their international nomenclature and origin.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Short Name</th>
<th>Origin</th>
</tr>
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<tbody>
<tr>
<td>B6-Kras^{tm4.1Tyj,-Trp53^{tm1Bm}</td>
<td>Kras^{lsLstG12D/wt}, p53^{0fl}</td>
<td>Gift from Florian Kühnel, Central Animal Laboratory, Medical School Hannover (CTL MHH)</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>C57BL/6J</td>
<td>JAX-ID 000664, CTL MHH</td>
</tr>
<tr>
<td>NOD.Cg-Prkdc^{scid} IL2rg^{tm1Wij/SzJZtm}</td>
<td>NSG</td>
<td>JAX-ID 005557, CTL MHH</td>
</tr>
</tbody>
</table>

Kras^{lsLstG12D/wt}, p53^{0fl} is a mouse strain with a stop-codon flanked by LoxP-sites in front of the mutated Kras^{G12D} allele and a p53-gene homozygously flanked by LoxP-sites. C57BL/6J is an immunocompetent widely used inbred strain. NSG mice are an immunodeficient strain with a null allele of the Interleukin 2 receptor IL2 gamma chain and severe combined immune deficiency scid, a mutation in the DNA repair complex protein Prkdc. Therefore, the NSG mice are deficient of T-cells, B-cells and functional NK-cells.

8.1.2 Establishment of cell lines

To harvest the liver or a tumor, mice were euthanized with cervical fracture according to animal welfare regulations and tissues were processed according to published protocols.

8.1.3 Orthotopic Injection

Depending on the strain background of the cells, injections were either performed into immunocompetent (C57Bl/6J) in case of congenic cells or immunocompromised (NSG) mice in case of cells with mixed strain background. For subcapsular orthotopic injections into the liver mice were anesthetized with isoflurane inhalation (TVA 15/1945, Initiation Isofluran (5%
+ \text{O}_2 (5\text{l/min}) \text{ and maintenance Isofluran (2\%)} + \text{O}_2 (1\text{l/min}) \text{ or intraperitoneal injection of Ketamin (80 mg/kg), Midazolam, (0.7 mg/kg) and Rompun (2 mg/kg). The eyes of the anesthetized mice were protected by the use of Dexpanthenol. The mice were kept warm with a heat lamp before and directly after the operation to prevent body temperature reduction. Carprofen was applied subcutaneously (5 mg/kg) before narcosis to prevent postoperative pain. Additionally, for the first to the third postoperative day, Metamizol was added to the drinking water (0.8 ml to 500 ml water). For the subcapsular injection, a short substernal longitudinal incision was performed and the left liver lobe exposed. Organoids resuspended in a 50\% Growth Factor Reduced Matrigel (Corning)/ DMEM F12 Advanced (Gibco) solution were implanted using a 30-g Hamilton syringe. After needle-retraction, the injection site was compressed with a sterile cotton swap followed by a wash of the abdominal cavity with 2 ml of sterile, prewarmed water. The abdominal wall was closed layer-wise with the inner peritoneum with absorbable 6-0 sutures and the outer skin with 4-0 sutures. The duration of the whole procedure was approximately 5-7 min. Mice were monitored for signs of ill health or tumor development by inspection and palpation twice a week and scored according to an activity score (Van Griensven et al, Shock 2002; with modifications, see Table 3). When an abdominal tumor was palpable or a score of 3 was reached, daily controls were performed. Mice were harvested when they showed a score >3 by cervical dislocation. Experiments were terminated and mice euthanized by cervical dislocation after 6 months if no tumor development occurred.

8.1.4 Subcutaneous injection

For subcutaneous injections, cells were resuspended in 50\% Growth Factor Reduced Matrigel (Corning)/ DMEM F12 Advanced (Gibco) solution and injected into the rear flanks of recipient mice. 50 µl were injected per flank without narcosis. Mice were monitored for signs of ill health or tumor development by inspection and palpation twice a week and scored according to an activity score (Van Griensven et al, Shock 2002; with modifications, see Table 2). Tumor growth was followed with caliper-measurements. When a tumor diameter was >0.5 cm or a score of 3 was reached, daily controls were performed. Mice were harvested when they showed a score >3 by cervical dislocation. When a tumor was ulcerating, impaired the movement of the mice, growing infiltrating or reached a size with a diameter of 1.2 - 1.4 cm, mice were euthanized and the tumors harvested for further analysis. Experiments were terminated and mice euthanized by cervical dislocation after 6 months if no tumor development occurred.
8.1.5 Gemcitabine treatment

Mice were randomized into gemcitabine- or placebo-treated groups when the tumor reached a diameter of 0.5 cm. Gemcitabine 100 mg/kg or vehicle (NaCl 0.9%), were injected intraperitoneally twice a week (10 µl/g body weight). Mice were monitored for signs of ill health or tumor development by inspection and palpation twice a week and scored according to an activity score (Van Griensven et al, Shock 2002; with modifications, see below). Tumor growth was followed with caliper-measurements. When a tumor diameter >0.5 cm was detectable controls were performed every other day. When a score of 3 was reached, daily controls were performed. Mice were harvested when they showed a score >3 by cervical dislocation. When a tumor was ulcerating, impaired the movement of the mice, growing infiltrating or reached a size with a diameter of 1.2 - 1.4 cm, mice were euthanized and the tumors harvested for further analysis.

8.2 Manuscript II: Generation of focal mutations and large genomic deletions in the pancreas using inducible in vivo genome editing, Carcinogenesis 2019

All animal experiments were approved by the LAVES (animal protocol 15/1986).

8.2.1 Mouse strains

Table 4: Mouse strains used in Manuscript II, named with their international nomenclature and origin.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Short Name</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>STOCK Ptf1a&lt;sup&gt;tm1(cre)Cvw&lt;/sup&gt; Gt(ROSA)26Sor&lt;sup&gt;tm2(CAG-rTA3-mKate2)Slowe&lt;/sup&gt;Col1a1&lt;sup&gt;em(tetO-EGFP, Cas9, U6-sgCR8)&lt;/sup&gt;</td>
<td>KC-RIK-sgCR8</td>
<td>Max-Planck Institute for Biophysical Chemistry, Göttingen</td>
</tr>
<tr>
<td>STOCK Ptf1a&lt;sup&gt;tm1(cre)Cvw&lt;/sup&gt; Gt(ROSA)26Sor&lt;sup&gt;tm2(CAG-rTA3-mKate2)Slowe&lt;/sup&gt;Col1a1&lt;sup&gt;em(tetO-EGFP, Cas9, U6-sgRnf43-A)&lt;/sup&gt;</td>
<td>KC-RIK-sgRnf43-A</td>
<td>Max-Planck Institute for Biophysical Chemistry, Göttingen</td>
</tr>
<tr>
<td>STOCK Ptf1a&lt;sup&gt;tm1(cre)Cvw&lt;/sup&gt; Gt(ROSA)26Sor&lt;sup&gt;tm2(CAG-rTA3-mKate2)Slowe&lt;/sup&gt;Col1a1&lt;sup&gt;em(tetO-EGFP, Cas9, U6-sgRnf43-B)&lt;/sup&gt;</td>
<td>KC-RIK-sgRnf43-B</td>
<td>Max-Planck Institute for Biophysical Chemistry, Göttingen</td>
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<tr>
<td>STOCK Ptf1a&lt;sup&gt;tm1(cre)Cvw&lt;/sup&gt; Gt(ROSA)26Sor&lt;sup&gt;tm2(CAG-rTA3-mKate2)Slowe&lt;/sup&gt;Col1a1&lt;sup&gt;em(tetO-EGFP, Cas9, U6-sgRnf43-B)&lt;/sup&gt;</td>
<td>KC-RIK-sgDel-A/B</td>
<td>Max-Planck Institute for Biophysical Chemistry, Göttingen</td>
</tr>
</tbody>
</table>
KC-RIK-sgCR8, KC-RIK-sgRNF43-A, KC-RIK-sgRNF43-B and KC-RIK-sgDel-A/B were mice generated by morula injection at the Max-Planck Institute for Biophysical Chemistry in Göttingen and were kept in quarantine after transport to the CTL of the MHH before transferring them to the IVC area. For detailed description see Manuscript II.

8.2.2 Food

Autoclaved water and commercial pellet diet (Altromin 1324, total pathogen free, Germany) was available ad libitum. KC-RIK-sgCR8, -sgRNF43-A and -B mice were fed with dox-enriched food (625 mg/kg from Altromin, Lage, Germany) for 14 days and KC-RIK-sgDel-A/B mice for 21 days and were returned to commercial pellet diet afterwards.

8.2.3 Monitoring of the mice

Mice were monitored twice a week for tumors via palpation or signs of illness and scored according the modified Van Griensven Score. If an abdominal tumor was palpable or the score was 3, daily controls were performed. If a tumor with a size >1.4 cm³ was palpable or the van Griensven score was above 3, the mice were euthanized in accordance with animal welfare via cervical dislocation.

8.3 Manuscript III: Potent antitumor activity of liposomal irinotecan in an organoid- and CRISPR-Cas9-based murine model of gallbladder cancer, Cancers 2019

All animal experiments had been approved by the LAVES (TVA 18/2827) and were performed by colleagues. I was not involved in animal experiments and assisted in the project only with experiments performed in vitro.

8.3.1 Mouse strains

Table 5: Mouse strains used in Manuscript III, named with their international nomenclature and origin.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Short Name</th>
<th>Origin</th>
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<tbody>
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<tr>
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<td></td>
<td>MHH CTL</td>
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<td>NSG</td>
<td>JAX-ID 005557</td>
</tr>
<tr>
<td>IL2rg&lt;sup&gt;tm1Wjl/SzJZtm&lt;/sup&gt;</td>
<td></td>
<td>MHH CTL</td>
</tr>
</tbody>
</table>

8.3.2 Subcutaneous injection

Subcutaneous injections were performed as described in manuscript I.
8.3.3 **Orthothopic injection**

Orthothopic injections into the gallbladder were performed as described in manuscript I with some slight changes. The organoids were injected into the lumen of the gallbladder instead of the liver lobe in 10 µl of 100% Growth factor reduced Matrigel (Corning, NY) using a 31G syringe.

8.3.4 **In vivo chemotherapy treatment**

Organoids were implanted subcutaneously and tumor growth was monitored with caliper measurements twice a week. When the tumor size reached 150 mm³, mice were randomized and treated intravenously either with irinotecan (50 mg/kg) or Nal-Iri (25 mg/kg or 50 mg/kg) or vehicle (0.9% NaCl) once weekly. Mice were evaluated for signs of ill health or tumor development twice a week and scored according to an activity score (Van Griensven et al, Shock 2002; with modifications, see Table 2) as previously described in manuscript I.
9 **Aim of the work**

Pancreatic and hepatobiliary tract cancer are intestinal cancer types with an increasing incidence and high mortality, partly because of missing adequate models to study them. Firstly, there is an urgent need for a genetically flexible, rapidly generated mouse model reflecting the human cancer types in an immunocompetent environment. Second, genetic changes specific for each of the deadly diseases need to be studied, and basic research is necessary to improve treatment. As the CRISPR/Cas9 system gets more and more prominent in cancer research, it should be combined with the future perspective of 3D-cultured healthy liver cells or gallbladder cells for basic cancer research like tumor formation, cell plasticity and therapy.

**Aim 1: Establishment of an organoid based mouse model for Cholangiocarcinoma**

At first, the isolation and propagation of murine organoids from adult murine liver should be established. Additionally, the genetic manipulation of the healthy wildtype organoids with RNA interference and CRISPR/Cas9 technology, as well as the use of the cre/loxP system to activate endogenous mutational changes, needed to be implemented. The influences of these manipulations and characterization of organoids in vitro should be addressed. Conclusively, the transplantation into mice, either orthotopic or subcutaneous, should be performed to develop a murine cholangiocarcinoma (CCA) cancer model.

**Aim 2: Characterization of the murine CCA model**

The purpose of the establishment of a murine CCA model was to reproduce the human disease as accurately as possible, especially the hallmark of a strong desmoplastic stroma reaction. Therefore, a histological characterization of resulting tumors upon transplantation should be performed. The system should have the ability to functionally characterize candidate cancer genes also in regard to treatment studies, including the influence of the microenvironment and immune system. Another goal was to test the plasticity of the organoids by inducing liver cancer depending on the driving oncogene and, finally, the comparison to human CCA.

**Aim 3: Transfer of my expertise to further in vivo mouse models**

Furthermore, we paralleled the experimental set up to the establishment of gallbladder organoids and the characterization of the resulting gallbladder cancer murine model. Finally, with the knowledge gained, I supported the establishment of the CRISPR/Cas9 based murine model in combination with pancreatic cancer genetically engineered mouse model based on embryonic stem cells.
10 Summary of the published results

The established and characterized murine CCA model resulted in a publication in Hepatology Communication 2019 with the title "Murine Liver Organoids as a Genetically Flexible System to Study Liver Cancer In Vivo and In Vitro" where I retain the shared first authorship. Therefore, I mainly focus on this publication and the results I rendered as summarized here.

Moreover, I have supported and used my expertise for the accomplishment of two further publications: the organoid-technology and in vivo models to establish an organoid based mouse model for gallbladder cancer (GBC) (Manuscript submitted in Cancers, 2019, "Potent antitumor activity of liposomal irinotecan in an organoid- and CRISPR-Cas9-based murine model of gallbladder cancer" and a GEMM-ESC based mouse model of pancreatic cancer, published 2019 in Carcinogenesis titled "Generation of focal mutations and large genomic deletions in the pancreas using inducible in vivo genome editing."

10.1 Murine liver organoids as a genetically flexible system to study liver cancer in vivo and in vitro

10.1.1 Murine liver organoids show a biliary phenotype in vitro

For establishing liver organoids, we digested healthy liver from adult mice with different genetic backgrounds and plated these cells in matrigel covered with murine liver organoid media according to published protocols. FACS analysis of the cell suspension shows only a minor fraction of epithelial cell adhesion molecule EpCAM expressing cells (<1%), whereas liver organoid cultures are almost completely EpCAM positive (99%).

Liver organoids can be genetically manipulated via RNAinterference, CRISPR/Cas9 technology and overexpression of oncogenes. Transducing Kras\textsuperscript{G12D/wt}, p53\textsuperscript{fl/fl} liver organoids with a neomycin-selectable plasmid expressing an inducible Cre-recombinase activates the mutated KRAS and homozygous excision of p53, both confirmed by PCR. This endogenous mutational activation leads to faster-growing organoids in vitro than their wildtype counterpart without any morphological differences.

10.1.2 Pre-malignant liver organoids give rise to CCA in vivo

Transplanted subcutaneously into mice, the activated Kras\textsuperscript{G12D}, p53\textsuperscript{fl/fl} ("pre-injection") organoids form moderately differentiated adenocarcinomas with 100% penetrance. These adenocarcinomas histologically show biliary structures of Cytokeratin 19 (CK19) positive tumor cells surrounded by a strong desmoplastic stroma, which is a prominent characteristic of
CCA. Additionally, we stained abounding collagen-fibers with sirius red/fast green and cluster of differentiation (CD31) showing the present vascularity in the tumor and elevated mucin production indicated by alcian blue, reflecting the human disease accurately. Isolated cell lines from the tumor cultured in either 2D or 3D culture conditions (called "tumoroids") were further analyzed in comparison to the wildtype and pre-injected organoids. Comparing the MAPK-signaling, p44/42 MAPK (ERK) gets activated via phosphorylation at T202/Y204 after Cre-recombinase excision and mutational activation of KRAS, and even stronger in combination with p53 loss. Shown in western blot analysis, p21 is only faintly reduced expressed after KRAS-activation and its expression almost completely gone after p53 excision. In the tumor cell line derived from $Kras^{G12D} \; p53^{Δ/Δ}$ organoids, the ERK-phosphorylation is comparably strong, and no p21 is detectable.

Genomic copy number alterations showed an increased quantity of deletions and amplifications after KRAS-activation and p53-deletion and even more after tumorigenesis in vivo, analyzed in wildtype (wt) organoids (unexcised), pre-injection organoids and the corresponding tumoroids. Regions of the detected alterations retain oncogenic potential and are altered in human CCA patient samples.

Next, we compared the transcriptome profiles of the same samples in unsupervised cluster analysis. The expression profile between the wildtype and the pre-injected, cre-activated organoids are similar, and only the related tumoroids are markedly different. Gene set enrichment analysis showed that in the pre-injected organoids gene sets associated with the cell cycle regulation, E2F and myelocytomatosis oncogene MYC were activated, underlining the shown increased proliferation in vitro. After tumorigenesis in vivo, gene sets associated with inflammation and paracrine signaling are activated, leading to the hypothesis of these pathways being important during tumor progression.

10.1.3 Organoid derived tumor-cell lines are serially transplantable independent of their culture conditions

Intending the scalability of the system for drug testing, tumor cell lines from the organoid-derived CCA were established and cultured either in a 2D or 3D condition. Resulting tumors of these reinjected cell lines lead to comparable histology similar to the parental tumor. We detected no difference in the stroma content, and they remain as a G2 or G2-3 grade of differentiation. Therefore, genetically defined and easily expandable 2D cell lines should also be used for testing targeted treatment options. In vitro testing with the PI3K inhibitor BKM120 and the MAPKK inhibitor, selumetinib was performed. Analyzed by a cell viability assay
measured after 24 h and 48 h, both drugs inhibit the proliferation of the cholangiocarcinoma cell line \( \text{Kras}^{G12D}; \ p53^{Δ/Δ} \). As a combinatorial treatment, the response is additive, but not synergistic. The response of the two different cultured cell lines was comparable to BKM120, and selumetinib cell-cycle FACS analysis determined the cells staying in the G1/G0 cell cycle phase.

10.1.4 Validation of cancer drivers in CCA in vivo

Stable knockdown of the in cholangiocarcinoma commonly mutated tumor suppressor gene phosphatase and tensin homolog (PTEN) via RNAinterference coupled with GFP or a non-targeting control shRenilla was introduced in addition to the KRAS-activated and p53 deficient organoids. Transplanted into mice, the knockdown of PTEN accelerated the tumor growth significantly without histological changes and stage of tumor grading. The activation of the PI3K signaling pathway was proven to show activating phosphorylation on the T308 of AKT in the PTEN-downregulated cell lines. In immunohistochemistry, PTEN-staining showed a still strong PTEN-expression in the stroma but none in the ductal tumor cell structure. These are CK19 positive and contrarily to the PTEN-staining GFP positive. The stroma is displayed GFP negative as it consists of recruited cells from the recipient mice. Concluding, we proved the ability of the system to validate candidate cancer drivers in CCA-carcinogenesis. Next, we wanted to investigate if the found accurate presentation of CCA dependents of the place of transplantation, we injected organoids \( \text{Kras}^{G12D}; \ p53^{Δ/Δ}; \ shPten \) intrahepatic. We obtained comparable CCAs with no differences in stroma content between subcutaneous and intrahepatic location.

The CRISPR/Cas9 system is a technology facilitating genetic manipulation. We transfected \( \text{Kras}^{lslG12D/wt} \) organoids with a plasmid expressing a Cre-recombinase, Cas9 and two sgRNAs targeting \( p53 \) and \( Pten \). In a T7 endonuclease assay, the sgRNA-induced gene cleavage was detected for \( p53 \) and \( Pten \) in pre-injected organoids, as well as in resulting tumor cell lines, and the protein level of \( p53 \) and PTEN supports these results as seen by western blot. This syngeneic \( \text{Kras}^{G12D/wt} \) organoids can be injected into immunocompetent C57BL/6J and give rise to CK19-positive, well-differentiated CCA.

First-line chemotherapy in cholangiocarcinoma is gemcitabine. We investigated the benefit of Gemcitabine treatment on the tumor development in our murine immunocompetent model representing the human diseases with the main hallmark of a strong desmoplastic stroma reaction. Gemcitabine treatment of CCA-bearing mice leads to a moderate survival benefit of 32 days compared to 42 days in the vehicle control group, but no complete tumor regression.
This is matching the patient's response, proving that the *in vivo* organoid-based mouse model is suitable for preclinical drug testing.

10.1.5 Liver organoids can give rise to CCA and HCC

Next, we wanted to investigate if the biliary phenotype of the organoids *in vitro* affects CCA development. Substituting the oncogene from *Kras* to myelocytomatosis oncogene (*Myc*), we established C57BL/6J organoids. We transduced them with retroviruses coding for *Myc* coupled with a red fluorescent reporter (mCherry) in combination with RNA-interference mediated p53 knockdown (shRNA co-expressing GFP) and gene alteration of adenomatous polyposis coli *Apc* via sgRNA. Transplanted into mice, these organoids give rise to tumors with completely different histology: Tumor cells grow in a solid pattern in nests without prominent desmoplasia, histologically presented like hepatocellular carcinoma. Tumor cells are CK19 negative but glutamine synthetase positive and GFP positive, in contrast to the stroma. Concluding, organoids having a biliary phenotype *in vitro* keep their plasticity to give rise to a broad differentiation spectrum into CCA and HCC.

We performed RNA sequencing, and unsupervised cluster analysis revealed marked differences between HCC and CCA tumoroids. Gene-enrichment analyses validated that CCAs driven by the activation of KRAS-target genes, whereas HCC leads to an activation of the MYC-driven pathways. When comparing the gene expression between the wildtype organoids, pre-injection organoids and tumoroids of the HCC, they showed similar results than the CCA-approach. Pre-injected organoids and wildtype organoids cluster together, whereas tumoroids differ in terms of gene expression. Treatment with the MAPK-inhibitor selumetinib and the Pi3K inhibitor has almost no effect on the proliferation of the established HCC-tumor cell lines, indicating the differences in the tumor-driving signaling pathways.

Finally, a comparative analysis with a previously published data set of 70 human HCCs, 13 CCAs and seven liver cancers of mixed HCC/CCA histology confirmed that the transcriptome profiles of this organoid-based murine cholangiocarcinoma and hepatocellular carcinoma like tumors closely reflect the profile of corresponding human cancers.

This concludes the main advantage of the organoid-based murine mouse model, which we developed for liver cancer: closely reflecting the characteristics of the human disease in terms of histology, stroma content and transcriptome profile. Furthermore, the system is targetable using fluorescent proteins coupled to oncogenic regulators and can be implemented in an immunocompetent environment with high flexibility in genetic alterations due to a broad range
of techniques. Future tests for targeted treatment are simplified with the chance of scalability and comparable response to humans.

### 10.2 Potent antitumor activity of liposomal irinotecan in an organoid- and CRISPR-Cas9-based murine model of gallbladder cancer

In the recently submitted manuscript that I have co-authored, we show that gallbladder (GB) organoid cultures can be used to model gallbladder cancer *in vivo*. In this work, I supported the paralleling of the experimental designs and technical procedure that I established in the CCA organoid system and recapitulated how organoids can be used for cancer acceleration studies and the functional annotation of unknown cancer drivers also in the most aggressive biliary tract cancer, GBC. Histologically, GB organoids transplanted into mice recapitulate the feature of the human disease, including a strong stroma reaction, and half of all orthotopic tumor-bearing mice obtained metastasis in the lung, typical for GBC patients. Beyond using *Kras* as an oncogenic driver and a proof of principle of the system showing tumor acceleration through the gene interruption of *Pten*, this work highlights the role of *ERBB2* mutants in GB-carcinogenesis. Transferable also here is the retaining plasticity of the organoids and development of different cancer-subtypes depending on the oncogenic driver. Tumors driven by activated *Kras* lead to adenocarcinomas, whereas mutant human *ERBB2* leads to papillary gallbladder cancer. Besides, we expand on pharmacodynamic studies to show that the system is suitable to determine the concentration of clinically relevant therapeutic agents, exemplified by Nal-IRI. The liposomal formulation of irinotecan increased the survival benefit more than irinotecan itself and reached with a significantly higher concentration the epithelial tumor cells than the stroma. Specifically, I established the culturing of the parental gallbladder organoids from mice, cloned the Cre, Cas9 and sgRNA containing plasmids and specified the cell of origin with EpCAM-FACS analysis, with a significantly higher number of EpCAM-positive cells in the gallbladder (21.1%) compared to the liver. Whereas gallbladder organoids are almost completely sustained of EpCAM-positive cells.

### 10.3 Generation of focal mutations and large genomic deletions in the pancreas using inducible in vivo genome editing

Another way for producing genetically engineered mice with a complex genotype without time-consuming breeding is the use of engineered embryonic stem cells, manipulated *in vitro* and injected into the morula for producing mice cohorts. Multi-allelic embryonic stem cells are
targeted with regulatable CRISPR/Cas9 constructs which lead to organ-specific and inducible genome editing, detailed described in Saborowski et al., Genes & Development 2014. The system used here enables expression of a Cre-recombinase co-expressing a red fluorescent-protein mKate which activates latent mutant Kras and doxycycline-inducible CRISPR/Cas9 technology only in exocrine pancreatic cells. In this work, we could show that loss of RNF43 leads to reduced survival and increased tumor development. Specifically, two different sgRNA-Plasmids targeting Rnf43 were cloned, which in the ESC-based mouse cohort in combination with activated KRAS both lead to invasive PDAC-formation of 57% and 75% compared to 28.6% in the control group. In 49.58% of all mKate positive cells, 35.89% showed effective Rnf43-cleavage, displaying the efficiency of the CRISPR/Cas9 system.

In addition, the expression of dual sgRNA constructs targeting the regions surrounding the Ink4a/Arf locus leads to the generation of homozygous deletions spanning 1.2 megabases and development of pancreatic cancers with several tumor foci with full penetrance. Resulting tumor cell lines are a heterogeneous population, due to repair mechanism indels were introduced. As the Cas9 is active throughout the entire pancreas, the model might be particularly well suited as an experimental model system for the generation of large homozygous deletions. Leakages in the system were not found, and the inducible Cas9 lowers the risk of off-target effects and makes a complete loss-of-function phenotype in mice.

The embryonic stem cell genetically engineered mouse model system is highly flexible. It reflects the human dismal prognostic disease pancreatic ductal adenocarcinoma in the environment of a non-mutated stroma and intact immune system.
11 Discussion

11.1 CRISPR/Cas9, ESCs & organoids for modeling cancer – genetic tools of the future?

11.1.1 Is the use of murine models still necessary?

Even though, a mouse is a different organism than a human with dissimilarities in tumor development and treatment, in vivo models in mice recapitulate the important hallmarks of cancer and support the investigations of the pathobiology and treatment response. Different mouse models keep their specific advantages in the study of either cell transformation, pre-neoplastic lesions, tumor maintenance, the tumor environment interplay or metastasis. Still, most of the mouse models, especially in gallbladder cancer (GBC), pancreatic adenocarcinoma (PDAC) and cholangiocarcinoma (CCA), lack the accurate presentation of the human disease, especially an appropriate tumor-environment with subsequent effects on therapy. Next to the differences in the organism, drawbacks of in vivo models are the high cost, especially in genetically engineered mouse models (GEMMs), and the length of time establishing these models. Organoid-based mouse models and ESC-based GEMMs circumvent at least partly these drawbacks – as the production is fast, but the cost of husbandry and maintenance of mice remain.

Recently large volumes of data showing genetic alterations in PDAC, GBC and PLC are published, but the functional annotation in combination with an organismal environment is of greater importance.13,19,35,36,38,41,44 Specifically, which genetic alterations are driver mutations, which are only passengers and relevant in terms of therapy or metastasis. Therefore, the flexibility available in the ESC- and organoid-based murine model is useful. In these three established models, only genetic alterations are considered as influencing factors for tumor formation. Risk factors like inflammation of the pancreas or especially the liver or gallbladder or metastatic diseases, have a high impact on tumor development,159 but were not considered in the models established in this work. The ESC-and organoid-based model in this work only considers genetic changes guiding tumor development without any organ damage, but further development could also include these risk factors in combination with the genetically flexible murine models.

In traditional transgenic mouse models, tumor development starts endogenously, and gradual changes within the epithelial tumor cell compartment along with the surrounding stroma happens stepwise. This allows the process of early tumor-stages and the possibility to follow up on this progression. An ESC-based mouse model with inducible genetic manipulations in
adult mice makes this possible, especially when the manipulation can be introduced in a physiological, adult mice age. Our developed pancreatic mouse model enables that – Dox-induction of the expression of the Cas9 can genetically change the pancreas-genome at the desired time point. Here, precursor lesions can be found easily with tractable genetic manipulations that are coupled with fluorescent proteins. In contrast, premalignant organoids are injected with the final tumorigenic potential into a healthy environment, incompletely mirroring the tumor formation process.

For liver and gallbladder and their cancer types, not many in vitro systems are available. Culturing primary hepatocytes in traditional culturing methods is difficult, they do not proliferate and lose their functions quite fast. Therefore, studying hepatobiliary cancer starting from an untransformed cell is not possible. Organoids overcome this disadvantage – pancreas, liver and gallbladder cells from an untransformed, "wildtype" or already genetically modified mouse can be cultured and used as starting material for the production of in vitro premalignant cells with a defined genetic background which are stable over the long term in culture and manipulatable with all known genetic modification-methods (CRISPR/Cas9, RNAi, stable gene overexpression). Even though available 2D human or murine cell lines of GBC, PDAC, CCA, or HCC are as described in the introduction, adapted to the specific culture conditions and, therefore, under selective pressure. In addition, they do not reflect the phenotypic profile of the parental tissue anymore. Therefore, in this work, we showed high similarities in the transcriptome profile of tumoroids established from murine cancer compared to human cancer (Hepatology Communications, 2019).

In vitro systems developed more recently in combination with xenotransplantation of human cell lines or patient-derived tissues into immunodeficient mice. However, these models do not recapitulate all hallmarks and especially not the complexity and crosstalk of carcinogenesis within an intact organismal environment and immune system. Especially these disadvantages get addressed in this work in the developed murine models – all three tumor models enable to study carcinogenesis in an immunocompetent environment with a strong desmoplastic stroma reaction. This is not only a characteristic of CCA, GBC and PDAC but also plays an important role in the drug response. As tumor-treatment drives more and more into the light of recent advances of immune therapy, it poses an advantage. Thus, the orthotopic injection and tumor formation might be important, as it displays best the available surrounding cell populations. Injection of human tissues occurs mostly subcutaneously in immunodeficient mice. Still, we could not show any differences in tumor development of the organoid-based tumor model in
CCA, but the metastatic level differed in the GBC-organoid based model. Therefore, the recruiting of cells might be comparable, but vascularity impacting the metastasis not.

Additionally, the genetic flexibility and environmental characteristics are not only important for the development of targeted therapy, but also essential in finding explanations in the clinically frequently occurring resistance and relapse even in precise oncology therapy. Properties of chemotherapeutic agents often show two problematics, the short half-life and small therapeutic index. Olive et. al hypothesize, that the poor perfusion of pancreatic cancer shown in human tumors and well displayed in KPC models limits drug delivery. In pancreatic tumors, various mouse models respond differently on gemcitabine treatment. Transplanted pancreas tumor cell lines in mice show almost no tumor growth upon gemcitabine treatment, on the other hand, gemcitabine treatment has an only little inhibiting effect on the increasing tumor volume in the KPC mouse model, which is consistent with the human response on gemcitabine treatment of patients harboring PDAC.\textsuperscript{162} Only 5-10\% of patients treated with gemcitabine demonstrate a response of decreased tumor size at the primary tumor site.\textsuperscript{163} The active metabolite dFdCTP did accumulate in the tumors of the transplantation models, but not in KPC-mice tumors. Blood vessels are significantly lower in tumors of the KPC model compared to transplantation models with less drug delivery into the tumor but increased delivery into the surrounding tissue. The Inhibition of the Hedgehog-pathway lead to reduced stromal myofibroblasts and following the gemcitabine delivery within the tumor in KPC tumors was elevated with improved survival and decreased metastasis to the liver.\textsuperscript{162}

As PDAC has many features in common with CCA, especially the hallmark characteristic of a desmoplastic stroma, responses to treatments are often similar. Chemotherapy with gemcitabine in combination with cisplatin is still the standard care of biliary tract cancer and no targeted therapy is available.\textsuperscript{26} Transplantation models often show, that gemcitabine almost completely stops the tumor growth in mouse models, which does not reflect the reaction of the patients. Therefore, we show in our organoid-based mouse model a reaction on gemcitabine comparable to the human response – gemcitabine slows tumor growth down but does not abolish liver tumor growth. It seems to reflect the pathologic drug transport to the tumor due to decreased vascularization (also shown as CD31- blood vessel staining in CCA tumors, which is there, but not of high amounts), suggesting this to be an important factor as it is in pancreatic tumor mouse models. Drug components which are even already in clinical phase II studies frequently do not reflect the human response on tumor-treatment with specific histology in xenografts. The predicted activity needs to be first translated from anti-proliferative activity \textit{in vitro} into mouse models \textit{in vivo} with a closest response to the human disease on drug
treatment.\textsuperscript{164} This is why our model is very valuable, it is important to find first \textit{in vitro} pre-clinical solid tumor models, with a response to drug treatment making a reliable prediction of the effect on a tumor with specific genetic and histologic context of the human disease. Therefore, a scalable system preferably with a broad range of genotypic phenotypes for drug testing which is not time-consuming or expensive is necessary. The comparison in this work (Hepatology communications, 2019) between organoid-derived tumor-cell lines cultured in either 2D- or 3D-conditions does not show a difference in the drug response \textit{in vitro} making it perfectly suitable for this purpose. This was surprising, as the 2D-cell lines compared to the tumoroids have no 3D-cell network contacts, assumed to be important in drug-testing models. A possible explanation might be a similar transcriptome profile or secreting factors of the tumor cell lines which also if re-transplanted into mice lead to a tumor comparable to the parental one independent of the pre-culture conditions. For irinotecan, GBC cells in culture need a significantly lower concentration to be effective than tumor cells in mice, indicating there a role of the environment (Manuscript accepted in Cancers).

The MAPK- and PI3K-inhibitors act on the activated pathways in the tumor and therefore change the cell cycle and proliferation range, but do not completely stop the cell proliferation (Hepatology Communications, Fig. 3). KRAS is the most frequent mutated gene in PDAC, CCA and GBC and results in sustained cell proliferation, but currently there is no direct anti-KRAS therapy available. Therefore, the downstream signaling needs to be suppressed and the two most prominent pathways hyperactivated are the MAPK pathway RAF-MEK-ERK and the PI3K pathway PI3K-AKT-mTOR. Problems of treatments are the development of new drugs which is expensive and resulting drugs mostly show adverse side effects. In our model with activation of the MAPK pathway due to mutation of Ras and loss of Pten activating the PI3K-pathway, a combinatorial treatment shows an additive effect, but not synergistically. The MEK inhibitor selumetinib was already shown as an inhibitor affecting proliferation and apoptosis in a Ras-activated mouse model\textsuperscript{141} and in B-cell non-Hodgkin lymphomas BKM120 treatment lead to mitotic arrest, in accordance to the cell cycle arrest we showed. Frequently, cancer shows resistance to BKM120 due to the upregulation of MEK, whereas the anti-tumor effect of BKM120 can be enhanced with MEK1/2 inhibition.\textsuperscript{165} Also in breast cancer combinatorial treatment of BKM120 with selumetinib showed promising therapeutic results.\textsuperscript{166} Unfortunately, the combinatorial treatment suppressing MAPK- and PI3K signaling does not fully stop cell growth even in cell culture conditions, where no stroma disturbs drug delivery. The high redundancy and crosstalk between the MAPK- and PI3K pathway often make therapy failing. In CCA cell lines regarding proliferation, apoptosis and cell signaling showed synergistic
effects when targeting mTOR or AKT in combination with MEK inhibition but also when targeting AKT and mTOR; showing that combinatorial treatment might be a viable approach for the future. On HCC-tumor cell lines, we could barely show an effect of selumetinib and BKM120, explained by the not directly activated MAPK- and PI3K pathway. HCCs treated with sorafenib often show a high phosphorylation level of ERK. Therefore, selumetinib could potentiate the anti-tumorigenic effect of sorafenib in HCC but as shown in our model, alone it does not have a direct effect on cell lines with a knock-down of p53, overexpression of MYC and a disrupted Apc expression.

In metastatic PDAC, Nal-IRI treatment as a second-line treatment after gemcitabine is already in clinical trials. There they show that a combinatorial treatment of Nal-IRI with 5-FU/LV prolongs survival of 4.5 months compared to 5-FU/LV treatment alone. Tumor-associated macrophages possibly play a role in the effectiveness of the drug, they might take up the drug and interfere with the transport. Comparable to our results in GBC, Nal-IRI has improved transport compared to irinotecan and therefore higher efficacy in the tumor, but still shows adverse side effects. The involvement of the stroma restraining irinotecan from tumors show the significance of studying the role of stroma in tumors. Either the drug transport towards the tumor cells and specificity of the drug delivery needs to improve or the stroma density reduced as well as increased vascularization of the tumor is needed to enhance drug response. The role of macrophages in the treatment of GBC need to be investigated, co-treatment of macrophages can even enhance Nal-IRI delivery to the tumor.

Two things need to be kept in mind for future treatment-development for PDAC, BTC and CCA: First, the desmoplastic, hypo-vascular stroma has a high impact on treatment effectiveness. Therefore, the stroma might need to be additionally targeted. Secondly, the heterogenic mutational landscape needs molecular profiling of patient tumors for personalized treatment options.

11.1.2 CRISPR/Cas9 as fast and efficient technology

Comparing the efficiency of gene disruption between the CRISPR/Cas9 technology and the Cre-based loxP technology is difficult. CRISPR/Cas9 convinced with its short time needed for cloning and its easy introduction into an organism. However, DNA repair happening after Cas9 cutting generates a heterogenous population of cells with genetic mutations making it therefore unpredictable. To introduce one or two sgRNAs and a Cas9 in combination with a Cre-recombinase at the same time, we transiently transfected cells with a plasmid coding for each with an individual promoter, named LCC sgp53 +/- sgPten (PP). As LCC does not contain a
selection marker, we co-transfected the cells with a GFP-Blasticidin-resistance cassette containing plasmid, to know the transfection efficiency and make them co-selectable. The GFP expression is not stably integrated or coupled, therefore does not necessarily need to be expressed or the sequence integrated. The transient expression of sgRNA, Cas9 and Cre can cleave the DNA during the time of transient expression, but due to the limited expression, it should have low side effects and a weak possible immune reaction against the Cas9. The duration of the Cas9 expression can control the altering of genes reducing, on one hand, the on-target mutation but also off-target effects and toxicity, which can occur with constitutive expression of the Cas9 and sgRNA expression. Therefore, we used this system to produce CCA and GBC in immunocompetent mice.

Successful CRISPR/Cas9 genome editing is dependent of the guide RNA sequence, algorithm publicly available on websites calculate a high on-target activity in addition to an as low as possible off-target prediction. In vivo sgRNA efficiency can vary from one gene to another. The sgRNA targeting Tp53 and Pten in this work originated from Xue et. al, Nature, 2014. They co-transfected NIH3T3 cell lines with Pxn sgPten with a GFP-containing Plasmid and 20% GFP positive cells were detected but 36.4% indels in Pten. Thus, assuming that the delivery of sgPten seems to be more efficient than GFP and sgPten efficiently mutates the Pten gene. Comparing sgPten delivered mice via tail vein injection with Ptnfl/fl mice with Cre recombinase delivered expressed by adenovirus did show similar knockout-rate of Pten. Most cells which got indels due to sgPten cutting also showed a complete mutation of all Pten alleles and therefore complete loss of the gene. Top-ranking sgPten off-target genomic sites of the mouse genomes were identified, as sgRNA can also detect and tolerate sequences with mismatches. None of the off-target sites of the top four ranked sites got cut detected by surveyor assay or deep sequencing, revealed at least less than 0.1% cleavage at the top off-target sequences and 10 surrounding nucleotides. The sgRNA targeting p53 used in this work was tested in similarly in NIH3T3 by the group around Xue et. al and showed 44.49% indel frequency. Co-injection via hydrodynamic tail vein injection of sgp53 and sgPten introduced 4% of Pten indel formation and 6.4% in p53 and lead to tumor formation after 3 months, comparable with Ptnfl/fl, p53fl/fl conditional knock out mice after adenoviral Cre-injection. In our approach in the LCC-PP-Plasmid, sgRNA against sgp53 and sgPten is expressed each under a U6 promoter in series. For testing the cleavage of the sgRNA, the T7 endonuclease assay shows clear results.

Next-generation sequencing was performed to find indel-frequency of LCC-sgp53 and LCC-PP (sgp53 and sgPten) in pre-injected gallbladder organoids. LCC sgp53 alone leads to an indel
formation of 36%, in the combinatorial transfection p53 got indel formations of 71.2% and Pten 90.2%. We did not check transfection efficiency, as we after selection assume almost 100% positivity, but initial transfection might have been different. In the tumor cell lines, indel formation detected in almost 100%. Even though, Xu et al showed more efficiency of the sgP53 than sgPten. Here, we did not check effectiveness from sgPten alone but paired sgRNA achieved higher indel formation for sgPten than sgP53. This can have different reasons, for example sgRNAs have varying efficiency depending on the cell type due to the chromatin packing and the resulting different reachable target sequence. Also, the position of the sgRNA sequence in the plasmid might change the expression strength. Still, the majority of cells got an indel formation in both target genes and as it should have a positive selectable effect due to their tumor suppressor function. The cells with interrupted p53 and Pten expression will grow out and form tumors. We did not detect how long cells express Cas9 and sgRNA, as they were just transiently expressed, they might get rid of the plasmids already after a few passages in vitro or spontaneously stably integrate some into the genome.

sgRNA against Rnf43 in the ESC based model constantly expressed sgRNA either against the control sequence CR8 or Rnf43-A or B and two days before birth the Cas9-expression was induced for 14 days in all mKate2 positive cells. Now investigating the efficiency of the sgRNA targeting Rnf43, ICE analyses were performed on DNA obtained from tissue sections and compared with the area of mKate2-positivity. The mean of 49.58% cells of the tissue did express Cas9 and 35.89 % of the whole tissue was edited in the Rnf43 gene. Both sgRNAs showed similar results and sgCR8 mice had 52.88% mKate2+ area and 29% effective gene edits. Between the tumors, the variety is small. All these results show a quite efficient CRISPR/Cas9 system which seems to be restricted to the pancreas as there were no mKate positive cells found in the lung or liver. Still, the CRISPR/Cas9 system in vitro in a limited time range does not lead to 100% genetic alteration in the target sequence, even though ESCs were selected for excision and Cas9 expressed for a long duration. This can be possibly explained by the repair-machineries of the cells. Therefore, sgRNAs targeting a negative selective pressure in a cell population might escape the gene targeting, also shown in an electroporation based screening method.174

In another approach, 13 sgRNAs targeting tumor suppressors of pancreatic cancer and two neutral control sgRNAs showed indel frequencies between 27% and 64% all simultaneously electroporated in the pancreas. Numbers of cells reached via electroporation is low, but all tumors had high-frequency indels at multiple of the sgRNA-targeting sites, maybe reflecting the CRISPR/Cas9-driver mutations give rise to cancer.150 A multiplexed CRISPR/Cas9 delivery
approach in liver was the introduction of multiplexed sgRNA via hydrodynamic tail vein injection, using the sleeping beauty transposon system. There, first a transient expression from the episomal plasmid and then long-term expression from genome integrated vectors should take place. Even though, none of the resulting tumors had stably integrated Cas9, which might be another hint for the toxicity in an organism. Ten sgRNAs targeting ten different genes injected induced mean mutant read frequencies of 4% of each targeted DNA site, increasing up to 40% due to positive selection of the oncogenic potential.\textsuperscript{174}

In general, as in the described sgRNA models, the CRISPR/Cas9 system does very effective alter genes even when the expression is time-limited.

11.2 Cell of origin, genetic background and the environment determine cancer characteristics

Organoids can grow out of almost any adult tissue including diseased tissues. For establishing GB or liver organoids, a piece of tissue (in the case of the murine GB, the whole organ) was digested to reach a single cell suspension which is plated entirely. To address the question, what kind of cells form the organoids, we performed EpCAM FACS-analysis on the single-cell suspension and the organoid-culture. (Hepatology Communications Fig. 1B, Cancers, Fig. 1D) We could show, that even a small amount of less than 1% positive EpCAM cells in the liver seems to be efficient for building organoids, which are almost completely EpCAM positive for liver and GB-organoids. These results are consistent with the literature, which describes that EpCAM negative cells do not form organoids,\textsuperscript{115} guiding the suggestion of a biliary phenotype as originating cell population. It is also shown, that the stem-cell marker Lgr5 positive cells are able to form organoids \textit{in vitro},\textsuperscript{175} which combined with our results might suggest a biliary progenitor cell as organoid-forming population. The liver has regenerative potential due to the stem-cell like population which is even bigger in the GB. Consistent with the higher amount of EpCAM positive cells (21.1%) in the GB than in the liver, the observation that GB-organoids have a higher establish-efficiency might be explained.\textsuperscript{121} This is also useful in regard that the GB is not an essential organ and therefore can be operatively taken and used for the establishment of organoids, where they can also be differentiated into hepatocytes and engraft either allogenic or autologous.\textsuperscript{117}

Several publications cover the question of cell of origin, CCA can arise from hepatoblast, hepatocyte and cholangiocytes, whereas HCC can arise from hepatoblast and hepatocytes. Back in the 1950s, a population of small proliferative oval-shaped cells in the adult liver was found
to contribute to the regeneration of both – cholangiocytes and hepatocytes. The origin of these
cells is the ductal compartment of the liver, expressing markers like Sox9 and Lgr5.\textsuperscript{113,117} We
did not check the expression of Lgr5 specifically, but liver and GB organoids do express SOX9,
a stem cell marker, and both express CK19, showing the biliary phenotype with a progenitor
status as the cells form organoids \textit{in vitro}. If the stem-cells alone build organoids in culture or
the factors in the medium lead to the biliary, stem-cell like character, is not completely clear.
Still, organoids are keeping their plasticity to result in different tumor types dependent on the
 genetic context – either CCA and HCC or tubular GBC and papillary differentiated GBC. On
 GBC, the most common histologic subtype is the adenocarcinoma, followed by the squamous
 subtype and the least frequent one, the papillary subtype (5-6% of all GBC). Due to its rarity,
 the literature does not provide much information. The survival outcome is worse for
 adenocarcinoma than the papillary subtype and seems to depend on different risk factors.
 Therefore, one study showed a correlation between mutated \textit{KRAS} and pancreatobiliary reflux
 forming papillary GBC, contrary to our finding where KRAS-activation leads to
 adenocarcinoma, but without any environmental damage. As these studies have very small case
 numbers and we did not use any induced inflammation in our mouse model, these risk factor
 might be necessary for developing papillary GBC in combination with KRAS\textsuperscript{G12D}.\textsuperscript{176}
 Concluding, our observation of developing different tumor types depending on the active
 oncogene shows another aspect in the GBC-research.

 This leads to the already long discussed question of the cell of origin for CCA and HCC. Here
 we can demonstrate that both, HCC and CCA arise from the same organoids depending on the
 genetic context (Hepatology Communications 2019). Even though common oncogenic
 pathways in PLCs are activated, some heterogeneity affecting the therapeutic targeting might
 be originating due to the cell of origin. Thus, this knowledge should be considered for individual
 treatment approaches and its precise effectiveness. PLCs have based on genomic analyses
 overlapping neoplasm rather than entirely distinct entities.

 The basic hepatic structure consists of parenchymal cells, which are the hepatocytes and
 cholangiocytes both arising from the same progenitor hepatoblasts. An obvious hypothesis is,
 HCCs arising from hepatocytes and CCA from cholangiocytes, heptoblasts can develop into
 both. There is one study underlining this hypothesis of PLC transforming cell-type dependent
 into CCA or HCC, where they developed mouse models with Cre-activated \textit{Kras} in
 combination with either homozygous or heterozygous floxed \textit{Pten} and cell type specific Cre-
 expression in the liver. Homozygous deletion of \textit{Pten} in hepatoblasts on an embryonic level
 leads to pure CCA development, heterozygous deletion to a mixed CCA/HCC population. In
mature hepatocytes of adult mice, tamoxifen induced Cre-recombinase expression under the control of an Albumin-promoter with homozygous or heterozygous Pten-deleted cells results always in HCC, but when induced earlier in younger mice, mice develop CCA. In the not mature young mice, Albumin-promoter are active in hepatocytes and in some bile ducts, which might form the CCAs. In adult mice, Alb-expression is restricted to mature hepatocytes which after Pten-excision transform into HCC. The Cre-recombinase expressed under the cholangiocyte specific CK19-promoter did not develop liver tumors but showed pre-malignant lesions in the liver with CCA-characteristics. The same genetic background, Kras activation and Pten loss can lead to HCC or CCA if transforming the corresponding cell type. Here, the activation of the PI3K pathway due to loss of Pten determines the fate towards biliary lineage during development, leading in combination with mutated KRAS to CCA. Progenitor/Stem cells are bi-potential cells, they can give rise to biliary ductal cells and hepatocytes and can drive hepatobiliary carcinogenesis into CCAs and HCCs. Stem cells as cells of origin in PLC is a marker for poor outcome for the patient, maybe because stemness traits are positively effecting tumor initiation, metastasis and therapy relapse.

Nowadays, there is evidence that the cell of origin of each cancer type is not determined but depends on their molecular feature. Lineage conversion during the malignant transformation is a possibility for developing CCA out of hepatocytes. Hydrodynamic tail vein injection (HTVI) mainly hits hepatocytes but cannot exclude plasmid DNA, also entering some bile duct epithelial cells or liver progenitor cells. HTVI delivered AKT-encoding DNA lead to a mixture of HCC and CCA combined with NICD overexpression even to CCA development. Notch-signaling, therefore, drives reprogramming and AKT accelerates the tumor formation. Both genes are frequently mutated and co-activated in human CCA, but if also in humans, hepatocytes give rise to CCA needs to be determined. The oncogene MYC drives HCC, whereas KRAS rather drives CCA, in our model independent of the Pten expression. Loss of p53 enables the expansion of progenitor-like cells and opens them for distinct cell fates through cooperating genetic events. This got confirmed with our liver organoid-based murine model, p53 loss seems to promote stem-cell characteristics, and the driving oncogene determines the fate of the initiating tumor. This might also explain different phenotypes seen in the KRAS- or ERBB-driven GBCs. The level of Myc expression has an impact on the stem-cell characteristic of the progenitor cells, and the self-renewal effect of MYC is dependent on p53.

Differentiated cells undergoing reprogramming with appropriate cell-type-specific pathway-activations may account for the phenotypic complexity in cancer. To summarize the possible ways from cell of origin to cancer, see Figure 3. Concluding that all cell lineages have the
potential of targeting for transforming events and contribute to hepatocarcinogenesis, they
might keep their biological traits of cellular origin in the tumor, influencing tumor initiation,
development and, therefore, treatment. Marker of the different cells of origin during the
diagnosis process would be very helpful.\textsuperscript{180} Taking all available sources of studies on the cell
of origin together, categorizing the development of liver tumors into CCA and HCC due to the
combination of a cell of origin and genetic context might be a misleading simplification
regarding all complex influences driving into cellular trans-differentiation especially affecting
neoplastic transformation.

Figure 3 Cell of origin of liver cancer. Stem or progenitor cells can develop HCC and CCA as
well as mixed phenotypes after pro-tumorigenic activation. Hepatocytes need reprogramming to
cholangiocytes in combination with proto-oncogenic changes for the CCA development, and
cholangiocytes need a lineage conversion to hepatocytes transforming finally into HCC.

Another study showed independence from oncogenic drivers but dependent on the micro-
environment, the development of HCC or CCA. Necroptosis-associated hepatic cytokine
microenvironment switches the fade from HCC to CCA, whereas suppression of the necroptosis
can revert that effect and lead to HCC. Transposable element stably delivered to the liver of
mice with loss of CDKN2A via HTVI further expressing oncogenic mouse MYC and
NRAS\textsuperscript{G12V} or murine MYC in combination with human AKT develop multifocal liver
carcinomas with an HCC-histology. Tumors developed from the same background, but
plasmids delivered with electroporation into the liver, lead to CK19-positive CCA with
differentiated hepatocytes as cells of origin for both tumor types. HCC and CCA show
epigenetically differences in the Tbx3-chromatin region and Prdm5, both transcription factors
described in roles in carcinogenesis. The environment, which is necroptosis, is characterized by
specific cytokines secreted from immune cells, which are activated by damage-associated
molecular patterns released from necroptically dying hepatocytes. The secreting factors of the environment might determine the development of HCC or CCA.\textsuperscript{159} As we developed HCC or CCA only via injection into undamaged healthy tissue either subcutaneously or orthotopically, we could not conclude anything from the organoid based model concerning the microenvironmental influence. But next to cholangiocytes and hepatocytes non–parenchymal cells are building the cells of the liver (fibroblasts, stellate cells, Kupffer cells and endothelial cells). They might play not a role in the cell of origin, but form a desmoplastic stroma, which is especially characteristic for CCA and GBC and the desmoplastic stroma reaction shown in the here described mouse models. Differences in availability and proximity of the cell of origin, cells of the microenvironment and the health conditions of the environment might determine tumor phenotype and aggressivity.

A few digestive tract cancers share common features like risk factors, either environmental or potentially genetic, why it often makes sense for a combined analysis or comparison may provide useful information. As PDAC, PLC and GBC share many genetic alterations and have some differences and (see Figure 1) originate from different cell types, it is still important to investigate the genetic change in every different cell type and the original tissue.
11.3 Outlook: Possibilities for cancer-research with the new developed models

ESC-GEMMs and an organoid-based mouse model with genetic changes, if needed tractable, with the overexpression of oncogenes (KRAS, MYC, ERBB), knockdown or knock-out of single tumor suppressors or deletions of complete chromosome regions give the full spectrum of tools reaching knowledge of the diseases especially on endogenous tumor development in adult mice including an appropriate environment and healthy immune system. In addition, they are fast and effective to produce (also due to CRISPR/Cas9), comparable cheap and reflect all characteristics of the tumors.

Appropriate tumor models for PDAC, HCC, CCA and GBC developed and characterized in this work serve as exemplary models for urgently needed investigations. In all tumor types, the impact of genetic changes represents a huge field to study as the tumor heterogeneity makes the full-understanding difficult. Therefore, the genetic flexibility should be used to find the driving effects of frequently mutated genes which are still functional unknown.

Additionally, our studies showed that stroma influences the treatment outcome in GBC and CCA and gemcitabine and irinotecan as wells as Nal-IRI do not cure the disease as it does not in humans. Therefore, targeted therapy and drugs targeting the stroma as well as combinatorial treatment of these should be studied on the developed appropriate models. In the beginning, basic research on factors introducing the strong stroma reaction should be investigated in detail on the PDAC, GBC and CCA model. The increased signaling of inflammation and paracrine signaling in CCA-tumoroids showed the importance of these signaling pathways in the tumorigenesis. Based on the RNA-sequencing data, which showed marked differences between HCC and CCA, tumor type-specific factors that influence the tumorigenesis in the mice could be identified and need to be tested for their impact on the stroma. The literature shows the impact of macrophages in Nal-IRI treatment, and these and other immune cell populations next to their impact on treatment require a closer look.
12 List of references


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15 List of Publications

2019
Zulrahman Erlangga, Katharina Wolff, Tanja Poth, Steffi Spielberg, Kai Timrott, Norman Woller, Florian Kühnel, Michael P. Manns, Anna Saborowski, Arndt Vogel, Michael Saborowski

Potent antitumor activity of liposomal irinotecan in an organoid- and CRISPR-Cas9-based murine model of gallbladder cancer
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Generation of focal mutations and large genomic deletions in the pancreas using inducible in vivo genome editing
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Murine Liver Organoids as a Genetically flexible System to Study Liver Cancer In Vivo and In Vitro
in Hepatology Communications

2016/09
Anne Hennig, Robby Markwart, Katharina Wolff, Yan Cui, Ian A. Prior, Manuel A. Esparza-Franco, Graham Ladds, Ignacio Rubio

Sequential engagement of Sos and feedback activation of neurofibromin account for transient Ras activation in response to growth factors in Cell Communication and Signaling

2015/01
Tobias Wagner, Nicole Kiweler, Katharina Wolff, Shirley K. Knauer, André Brandl, Peter Hemmerich, Jan-Herren Dannenberg, Thorsten Heinzle, Günter Schneider and Oliver H. Krämer

Sumoylation of HDAC2 promotes NF-κB-dependent gene expression in Oncotarget 2015


Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht. Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

Ort, Datum Katharina Wolff
17 Contribution of the candidate Katharina Wolff and all other authors

*Hepatology Communications, 02/2019*

*Murine Liver Organoids as a Genetically Flexible System to Study Liver Cancer In Vivo and In Vitro.*

Katharina Wolff, Anna Saborowski mainly did the data collection and data analysis.
Katharina Wolff, Anna Saborowski, Michael Saborowski mainly did data analysis and interpretation.

Anna Saborowski, Michael Saborowski, Arndt Vogel and Katharina Wolff planed the conception and design of the work and draft the article.

Steffi Spielberg, Benedikt Beer, Zulrahman Erlangga did some data collection and analysis.

*Carcinogenesis, 06/2019*

*Generation of focal mutations and large genomic deletions in the pancreas using inducible in vivo genome editing*

Amrendra Mishra mainly collect the data and performed analysis.
Katharina Wolff designed and cloned Plasmids, supported data collection and analysis and mice experiments as well as critical revision of the article.
Fatemeh Emamgholi, Zulrahman Erlangga performed data collection and analysis.
Björn Hartleben evaluated and classified histological slides and Kristian Unger analyzed the CGH-array. Michael Kessel and Ulrike Teichmann performed the ESC-injection.
Norman Woller, Lukas E. Dow, Florian Kühnel, Scott W. Lowe and Arnd Vogel advised experiment performing and data analysis. Michael P. Manns provided facilities, equipment and financial support.
Amrendra Mishra, Michael Saborowski and Anna Saborowski concepted and designed the work as well as draft the article.
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Potent antitumor activity of liposomal irinotecan in an organoid-and CRISPR-Cas9-based murine model of gallbladder cancer

Zulrahman Erlangga mainly collect and analyzed the data.

Katharina Wolff supported data collection and analysis, cloned Plasmids (LCC sgp53, CR8, PP) performed FACS analysis and cell culture based organoid work as well as establishing those from mice. Katharina Wolff critically revised the article.

Tanja Poth classified Histologies, Steffi Spielberg supported data collection and analysis especially in western blots.

Kai Timrott, Norman Woller, Florian Kühnel and Anna Saborowski advised experiment performing and data analysis. Michael P. Manns provided facilities, equipment and financial support.

Anna Saborowski supervised the work and critically revised the article.

Zulrahman Erlangga, Michael Saborowski and Arndt Vogel drafted the article, concepted and designed the work and analysed and interpreted the data.