In vitro modeling of pancreatic duct cell carcinogenesis

by

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Medical Biophysics

University of Toronto

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Abstract

Pancreatic adenocarcinoma (PDAC) putatively arises from the pancreatic duct, thus usage of the normal human pancreatic duct epithelial (HPDE) cell line is an ideal model to examine the successive accumulation of genetic alterations involved in carcinogenesis. KRAS mutations have been reported in 90% of PDACs. Oncogenic KRAS elicits activation of downstream pathways involved in survival, motility, and cell cycle progression. KRAS\textsuperscript{G12V} introduction in the HPDE cell line upregulates Lipocalin-2 (LCN2) expression. LCN2 has been identified in numerous carcinomas and is associated with survival, tumorigenicity, and invasion. In this work, LCN2 was found to be commonly expressed in high grade pancreatic duct neoplastic precursor lesions and PDAC illustrating its potential as a biomarker. Moreover, in vitro and in vivo studies demonstrate that high LCN2 expression promotes gemcitabine resistance, MMP-9 activity, angiogenesis, and tumorigenicity.

Loss of Smad4 function is found in 55% of PDAC cases. Smad4 is a critical component in the TGF-β signaling which mediates the transcription of genes involved in processes such as cell cycle arrest, apoptosis, and invasion. This work examined the consequences of KRAS\textsuperscript{G12V} expression and Smad4 loss in the HPDE model. Cellular invasion was promoted by KRAS\textsuperscript{G12V}
expression or knocking down Smad4 by 80% in the HPDE model. A TGF-β resistant HPDE cell line, TβR, was shown to lack Smad4 expression due to deletion, promoter methylation, and nonsense mutation. KRASG12V expression in the TβR model (TβR KRAS) promoted neoplastic transformation and tumour formation in immunodeficient mice with complete penetrance. Smad4 expression in the TβR KRAS cell line reinstated TGF-β signaling, delayed tumour formation, and decreased metastatic spread. This study provides evidence that Smad4 acts as a restriction point in the transformation of HPDE cells. Overall, this work examines the contribution of genes involved in transformation, and identifies a potential therapeutic and diagnostic biomarker in PDAC.
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<td>aCGH</td>
<td>Array comparative genomic hybridisation</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ARF</td>
<td>Alternate open reading frame</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>BAD</td>
<td>BCL-2 associated agonist of cell death</td>
</tr>
<tr>
<td>Bax</td>
<td>BCL-2 associated X protein</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix loop helix</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2 interacting mediator of cell death</td>
</tr>
<tr>
<td>BL2+</td>
<td>Biosafety level 2+</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>BNP3</td>
<td>BCL2/adenovirus E1B 19kDa interacting protein 3</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>Breast cancer susceptibility protein 1/2</td>
</tr>
<tr>
<td>CA19-9</td>
<td>Carbohydrate antigen 19-9</td>
</tr>
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<td>C3</td>
<td>Caspase-3</td>
</tr>
<tr>
<td>CC3</td>
<td>Cleaved caspase-3</td>
</tr>
<tr>
<td>CD31</td>
<td>Platelet endothelial cell adhesion molecule 31 or cluster of differentiation 31</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>CK19</td>
<td>Cytokeratin 19</td>
</tr>
<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Cross threshold</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted in colon cancer</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>eco</td>
<td>Ecotropic</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epithelial growth factor receptor</td>
</tr>
<tr>
<td>ELA</td>
<td>Elastase</td>
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<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
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<td>EP300</td>
<td>E1A binding protein p300</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FAMMM</td>
<td>Familial atypical mole-malignant melanoma</td>
</tr>
<tr>
<td>FANCD2</td>
<td>Fanconi anemia, complementation group D2</td>
</tr>
<tr>
<td>FGF7</td>
<td>Fibroblast growth factor 7</td>
</tr>
<tr>
<td>FKHR</td>
<td>Forkhead receptor</td>
</tr>
<tr>
<td>GADD45A</td>
<td>Growth arrest and DNA-damage-inducible, alpha</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDF</td>
<td>Growth and differentiation factor</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanine triphosphatase</td>
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<tr>
<td>H6c7</td>
<td>Human pancreatic duct epithelial 6 clone 7</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
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</table>
shRNA – Short hairpin RNA
SOS – Son of sevenless
st – Small t antigen
SV40 – Simian virus 40
TAK1 – TGF-β activated kinase 1
TβR – TGF-β resistant
TGFBR1, 2 – TGF-β receptor I, II
hTERT – Telomerase reverse transcriptase
TGF-β – Transforming growth factor beta
VEGF – Vascular endothelial growth factor
VHL – von Hippel-Lindau tumor suppressor
yr – Year

N.B. Any designation with the letter ‘m’ or ‘r’ before the term refers to mouse and rat, respectively, e.g. mPanIN refers to mouse PanIN lesions.
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Chapter 1

**Pancreatic function and development of cancer**

1.1 Pancreatic function and development

1.1.1 *Pancreatic anatomy and physiology*

The pancreas is located in the gastrointestinal tract which is situated in the abdomen between the stomach, duodenum, and spleen. The pancreas is a glandular organ of the digestive and endocrine system which is composed of several cell types. The exocrine portion of the pancreas is comprised of acinar and duct cells. The acinar cells synthesize, store, and secrete digestive enzymes to break down fats, proteins and carbohydrates. The digestive enzymes produced by acinar cells include amylase, carboxypeptidase, chymotrypsin, elastase, pancreatic lipase, and trypsinogen [1]. The duct cells form the epithelial lining of the ductules and main pancreatic ductal system which provides a drainage system for the secretions of acinar cells. In addition to its structural role, the duct cells also secrete ions, bicarbonate, and water which are necessary for optimal digestive enzyme function [2]. The endocrine portion of the pancreas consists of four specialized cell types which comprise clusters called the Islets of Langerhans. The α cells secrete glucagon, β cells produce insulin, γ cells secrete pancreatic polypeptide, and δ cells generates somatostatin. These hormones principally regulate glucose and lipid metabolism [3].
1.2 **Pancreatic cancer epidemiology**

1.2.1 *Epidemiology of pancreatic cancer*

Pancreatic cancer is the twelfth most common cancer type within Canada; however it is the fourth most common cause of cancer death. A diagnosis of pancreatic cancer is quite dismal as the number new cases are nearly equal to the number of deaths each year [4]. Ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer. Survival rates are low due to the lack of specific symptoms and poor response to non-surgical therapies. In the United States, the median age at diagnosis is 72 years old [5]. The 1-year survival rate is 19% and the 5-year rate is less than 5%. Only 10-20% of patients qualify for surgical resection due to the frequency of metastatic dissemination. Surgical resection increases the 5-year survival rate to 20% [6].

1.2.2 **Risk factors**

At present, the causes of pancreatic cancer remain unknown. Advanced age, cigarette smoking, family history of pancreatic cancer, pancreatitis, diabetes mellitus, obesity, environmental exposure, diet, and non-O blood group are risk factors associated with PDAC [7]. After adjusting for age, the frequency for pancreatic cancer is slightly higher in men than in women [5]. Approximately 5-10% of patients with pancreatic cancer have an underlying germline mutation which predisposes them to develop pancreatic cancer [5]. A family history of pancreatic cancer, germline mutations in BRCA1/2, and diseases such as hereditary pancreatitis, familial atypical mole-malignant melanoma (FAMMM) syndrome, Peutz-Jeghers, Li-Fraumeni, and cystic fibrosis have been associated with increased risk of developing pancreatic cancer [7].
1.3 Precursor lesions of pancreatic cancer

The majority of pancreatic neoplasms involve the duct and are known as pancreatic adenocarcinoma (PDAC). PDAC typically advances from well-defined precursor lesions termed pancreatic intraepithelial neoplasias (PanINs). Cystic precursors like mucinous cystic neoplasms (MCNs) and intraductal papillary mucinous neoplasms (IPMNs) have the potential to advance to PDAC. Other tumours that arise from the exocrine pancreas include pancreatoblastoma, acinar cell carcinoma, and solid pseudopapillary neoplasms [8]. Tumours of the endocrine pancreas are quite rare and account for 1-2% of all pancreatic cancer cases [9].

1.3.1 PanINs

PanINs are microscopic non-invasive precursor lesions that are less than 5mm in diameter. PanINs are composed of mucin-producing epithelia with varying degrees of cytologic and architectural atypia occurring in small pancreatic ducts. Normal ductal epithelium is composed of cuboidal to low-columnar cells (Figure 1-1). PanINs are sub-classified into PanIN-1, -2, and -3 lesions. PanIN-1A epithelial lesions display tall columnar cells without nuclear atypia. These cells have basally located nuclei and contain abundant mucin. PanIN-1B lesions have papillary or pseudostratified architecture, but are otherwise similar to the type 1A lesions. PanIN-2 lesions are papillary and have some nuclear abnormalities that include loss of polarity, nuclear crowding, hyperchromatism, and enlarged nuclei. PanIN-3 lesions are similar to type 2 lesions with additional cytological and architectural atypia. PanIN-3 lesions contain nuclear irregularities that are characterised by loss of nuclear polarity, prominent nucleoli, enlarged and hyperchromatic nuclei. These lesions are also identified by the budding off of small clusters of epithelial cells into the lumen or cribriforming, and dystrophic goblet cells [10,11].
Figure 1.1. *Molecular progression model of pancreatic cancer*. Pancreatic intraepithelial neoplastic (PanIN) lesions represent the evolution from normal duct epithelium to pancreatic ductal adenocarcinoma (PDAC). The temporal acquisition of common genetic aberrations is noted below. The frequencies of genetic alterations are based on observations made in ductal lesions. Scale bar represents 50μm (adapted from [12,13]).

1.4 Molecular genetics of PDAC

The development of the molecular progression model of PDAC couples the advancement of these histologically well-defined PanIN lesions with the acquisition of genetic aberrations in several cancer-associated genes (Figure 1.1) [13,14]. In the molecular progression model, activating KRAS mutations have been reported in 95% of cases. Following KRAS activation is the loss in function of the p16\(^{INK4A}\), p53, and Smad4 tumour suppressor genes in 95%, 75%, and 55% of PDACs, respectively [8,15].

1.4.1 KRAS

KRAS is a member of the RAS family of GTP-binding proteins which mediates a wide variety of cellular functions including cell-cycle progression, cellular survival, transcription, and cell motility. Mammalian RAS proteins are small membrane-associated guanine-nucleotide binding proteins encoded by three different genes which are translated into four highly homologous proteins HRAS, NRAS, KRAS4A, and KRAS4B. RAS proteins all contain a
highly conserved N-terminus and a C-terminus known as a hypervariable region. The hypervariable region contains residues which specify posttranslational modifications that target the RAS proteins to the appropriate cellular location. All RAS proteins are farnesylated at the terminal CAAX motif. Additionally, H-, N-, and KRAS4A are modified with palmitic acid upstream of the CAAX motif which anchors these proteins to the membrane [16]. Palmitoylation and depalmitoylation of H- and NRAS allows the proteins to cycle between the plasma membrane, Golgi apparatus, and endoplasmic reticulum [17]. KRAS4B has a stretch of lysine residues in the C-terminus which anchors it to the inner layer of the plasma membrane. KRAS4A and KRAS4B result from alternative splicing at the C-terminus. KRAS4B is the predominant splice variant and will be referred to as KRAS in this work [16]. Genetic studies in mice suggest that RAS proteins have redundant and unique roles. Loss of HRAS or NRAS alone or in combination is dispensable for normal mouse development [18,19]. Whereas, KRAS is essential for mouse embryonic development as its loss leads to anaemia and defective liver erythropoiesis [20,21].
Figure 1.2. **KRAS signalling pathway.** The binding of growth factors to cell surface receptors recruits adaptor protein complexes which catalyse nucleotide exchange to convert inactive RAS-GDP to active RAS-GTP. GTP-bound RAS activates downstream effector pathways such as MAPK and AKT (adapted from [16]).

RAS functions as a link between extracellular signals that communicate through receptor tyrosine kinases, cytokine receptors, integrins, and G-protein coupled receptors to activate downstream signalling events (Figure 1.2.). RAS acts as a molecular switch by cycling through its active GTP-bound state and inactive GDP-bound state. The binding of growth factors to receptors stimulates guanine nucleotide-exchange factors (GEFs) such as SOS. GEFs catalyse the exchange of GDP for GTP on RAS. GTP-bound RAS can activate downstream effectors including RAF, PI3K, and RAC. RAS proteins have low intrinsic GTPase activity which converts GTP to GDP. The rate of hydrolysis is enhanced by GTPase activating proteins.
(GAPs). GAPs and GEFs regulate RAS activity and stimulation of its downstream target pathways [22,23].

Active GTP-bound RAS triggers the RAF-MEK-MAPK cascade which promotes cell-cycle progression, cellular survival, transcription, and cell motility. Phosphorylated MAPK can interact with various transcription factors such as JUN and FOS in the nucleus. RAS-GTP also associates with PI3K which signals through the PI3K/PDK/AKT cascade. AKT promotes cellular survival through the phosphorylation of pro-apoptotic proteins BAD and FKHR. RAC activation by RAS-GTP promotes cell motility by regulating the actin cytoskeleton [16].

Mutated and constitutively active forms of RAS are found in 30% of all human cancers. HRAS mutations have been identified in bladder cancer. NRAS mutations have been reported in melanoma, myeloid leukemia, and liver cancers. KRAS mutations are found in biliary, colon, lung, and pancreas cancers [22]. Activating KRAS mutations appear early and occur in 95% of PDACs, which implicates a critical role for this gene as an initiating event [13]. Activating point mutations in codon 12 are among the most common genetic alterations found in PDAC [24]. These mutations impair the ability of GAPs to hydrolyze GTP which renders RAS constitutively active. Therefore, oncogenic RAS confers continual stimulation of downstream effector pathways which enhance cell growth, survival, and motility [16].

1.4.2 \textit{p16}^{\text{INK4A}}

The \textit{CDKN2A} gene encodes two tumour suppressor genes, INK4A and alternative open reading frame (ARF). Both gene products have independent first exons and share exons 2 and -3 which are transcribed from alternative reading frames. The \textit{CDKN2A} gene products negatively regulate cell proliferation. The \textit{p14}^{\text{ARF}} transcript stabilises p53 by blocking interaction with MDM2 which inhibits its proteolytic degradation. \textit{p16}^{\text{INK4A}} blocks entry into S phase by
interacting with CDK4 and CDK6 which impedes phosphorylation of RB. Maintaining RB in a hypophosphorylated state promotes its binding to E2F resulting in G₁ cell cycle arrest [25].

Loss of p¹⁶⁺⁵ᴷ⁴ᴬ function in PDAC occurs through a variety of mechanisms including intragenic mutation with loss of second allele (40%), homozygous deletion (40%), or promoter hypermethylation (10-15%) [13]. In PDAC, sporadic and germline mutations have been identified in exon1-alpha which encodes INK4A, but not exon 1-beta which codes for the p¹⁴⁺ᴬᴿ𝐹 transcript, however mutations or deletions in exon 2 target both p¹⁶⁺⁵ᴷ⁴ᴬ and p¹⁴⁺ᴿＦ [26-28]. p¹⁶⁺⁵ᴷ⁴ᴬ is dispensable for normal mouse development and tissue homeostasis. However these knockout mice are prone to the development of spontaneous cancers [29,30]. Forced CDKN2A expression in mice decreases tumour incidence compared to wildtype littermate controls illustrating its potency as a tumour suppressor [31].

1.4.3 p⁵³

p⁵³ is a homotetrameric DNA binding complex which activates the transcription of genes responsible for cell cycle arrest, apoptosis, DNA repair, and senescence in response to cellular stress. The majority of p⁵³ mutations are generally missense alterations that occur within the DNA binding domain, thus impairing its tumour suppressing function [32]. The p⁵³ tumour suppressor gene is mutated in 75% of PDAC cases [33]. The loss of p⁵³ function enables the growth and survival of cells that harbour tumourigenic genetic aberrations [32]. p⁵³ is dispensable for mouse development, however these knockout mice spontaneously acquire tumours [34,35].
1.4.4 *Smad4*

The Transforming Growth Factor-β (TGF-β) protein superfamily is comprised of many structurally related cytokines including Activin, bone morphogenetic protein (BMP), growth and differentiation factor (GDF), TGF-β, and Nodal. These cytokines play diverse roles in the regulation of cell proliferation, extracellular matrix protein deposition, cell motility, cell cycle, differentiation, and apoptosis [36]. In epithelial cells, TGF-β acts as a tumour suppressor by arresting growth. After malignant transformation, TGF-β promotes tumourigenic phenotypes particularly in the phenomenon called epithelial to mesenchymal transition (EMT). EMT is a process whereby epithelial cells take on mesenchymal phenotypes. Stimulation with TGF-β enhances these phenotypes by increasing migratory capacity, expression of extracellular components, invasiveness, and resistance to apoptosis [37].

In the canonical TGF-β signalling pathway, the TGF-β ligand first binds to TGF-β type-II receptor (TGFBR2) which recruits TGFBR1 (Figure 1.3). In the resulting receptor-cytokine complex, TGFBR2 phosphorylates TGFBR1 which then enables TGFBR1 to phosphorylate receptor activated Smad (R-Smad) proteins Smad2 and -3. Phosphorylation of R-Smads permits oligomerization with the common mediator of TGF-β signalling, Smad4. This association with Smad4 can occur in the nucleus or in the cytoplasm. Formation of the heteromeric Smad complex can initiate transcription of genes through the association with different cofactors such as the AP1, bHLH, forkhead, homeobox, and zinc finger families. Smad-cofactor complexes defines groups of expressed genes which coordinate processes including cell cycle arrest and extracellular matrix production [36].
Figure 1.3. *The TGF-β signalling pathway*. The TGF-β ligand binds to the Type II receptor which recruits the Type I receptor. The ligand-receptor complex results in the phosphorylation of Smad2/3. Phosphorylated Smad2/3 can then bind to Smad4. The Smad complex translocates to the nucleus where it interacts with DNA binding cofactors to regulate gene expression (adapted from [36]).

The Smad proteins are divided into three major groups. The R-Smads, Smad1, -2, -3, -5, and -8 are phosphorylated in response to receptor-cytokine complex formation. TGF-β, Nodal, and Activin activate Smad2 and -3, and binding of BMP and GDF to their receptors phosphorylate Smad1, -5, and -8. Smad6 and -7 are the inhibitory class of SMAD proteins (I-Smads) which act in a negative feedback loop to regulate TGF-β signaling. I-Smads impede TGF-β signaling by recruiting E3 ubiquitin ligases Smurf1 and -2 to TGFBR1, and obstruct R-Smad/Smad4 oligomerisation. Smad4 is the common Smad to all TGF-β ligands and complexes with phosphorylated R-Smads [38].
The Smad family of proteins contain highly conserved C- and N-terminals separated by a linker region. The N-terminal MAD homology (MH) 1 domain has DNA binding properties [39,40] and inhibits the functional activity of the MH2 domain [41]. Receptor mediated phosphorylation of the R-Smads occurs at the C-terminal motif Ser-X-Ser in the MH2 domain [42,43]. MH2 domain phosphorylation allows interaction with Smad4 and other cofactors which cause the transcription of downstream genes [44].

The TGF-β signalling pathway is frequently disrupted in pancreatic cancer. Loss of heterozygosity (LOH) of 18q is a common event that occurs in over 90% of pancreatic carcinomas. Biallelic loss of Smad4 is found in 55% of cases in pancreatic cancer. The loss of the second allele has been attributed to deletion in 35% and mutation in 20% [45]. Smad4 absence coincides with advanced neoplasia and has been correlated with increased likelihood of widespread metastasis [13,46]. Thus, Smad4 expression may be valuable for stratifying patients for different treatment strategies.

1.5 Animal models of pancreatic cancer

Genetically engineered mouse models to study human disease are commonly used to investigate the specific gene contributions. In the past decade, several pancreatic cancer mouse models have been developed which cover the most commonly observed genetic alterations [47]. Strategies to conditionally express specific genetic changes in the pancreas have exploited what is currently known about the developing mouse pancreas. The development of knock-in/knockout models depend on promoters that are responsive to genes expressed in the exocrine pancreas such as PDX1, p48, and ELA [48,49]. Despite species differences between mice and
humans, mouse models have provided a valuable research tool which complements studies done with patients and human derived cell lines.

1.5.1 **KRAS models**

The widespread embryonic expression of oncogenic KRAS is lethal so strategies were developed for targeted pancreatic expression [50]. KRAS$^{G12V}$ expression under the control of the duct specific cytokeratin 19 (CK19) promoter promotes ductal hyperplasia similar to what is observed initially in the progression of human PDAC (Table 1.1) [51]. Targeted KRAS$^{G12D}$ expression in acinar cells under the control of the ELA promoter caused acinar cell hyperplasia and dysplasia, as well as mPanIN lesions [48]. Under the control of the PDX1 or p48 promoter, KRAS$^{G12D}$ expression in embryonic pancreatic progenitor cells led to the development of mPanIN lesions which recapitulate human disease. A small percentage of these animals progress into fully invasive and metastatic disease [49]. Recently, a reversible KRAS$^{G12D}$ mouse model was reported. Oncogenic KRAS expression was required for the maintenance of mPanIN lesions and mPDAC [52]. These models illustrate that mutant KRAS expression is a tumour-initiating event and is required for sustaining pancreatic cancer, however further genetic changes are required for PDAC progression.
Table 1.1 **Mouse models of pancreatic cancer.**

<table>
<thead>
<tr>
<th>Model</th>
<th>Precursor lesion</th>
<th>PDAC</th>
<th>Median Survival</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK19-KRAS&lt;sup&gt;G12V&lt;/sup&gt;</td>
<td>no</td>
<td>no</td>
<td>--</td>
<td>Brembeck 2003</td>
</tr>
<tr>
<td>ELA-KRAS&lt;sup&gt;G12D&lt;/sup&gt;</td>
<td>mPanIN</td>
<td>no</td>
<td>~1 yr</td>
<td>Grippo 2003</td>
</tr>
<tr>
<td>Pdx1/Ptf1a-cre; LSL-KRAS&lt;sup&gt;G12D&lt;/sup&gt;</td>
<td>mPanIN</td>
<td>yes</td>
<td>~1 yr</td>
<td>Hingorani 2003</td>
</tr>
<tr>
<td>Pdx1-cre; LSL-p53&lt;sup&gt;R175H&lt;/sup&gt;</td>
<td>no</td>
<td>no</td>
<td>--</td>
<td>Hingorani 2005</td>
</tr>
<tr>
<td>Pdx1-cre; LSL-KRAS&lt;sup&gt;G12D&lt;/sup&gt;; p53&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>mPanIN</td>
<td>yes</td>
<td>3 mo</td>
<td>Bardeesy 2006</td>
</tr>
<tr>
<td>Pdx1-cre; LSL-KRAS&lt;sup&gt;G12D&lt;/sup&gt;, LSL-p53&lt;sup&gt;R175H&lt;/sup&gt;</td>
<td>mPanIN</td>
<td>yes</td>
<td>5 mo</td>
<td>Hingorani 2005</td>
</tr>
<tr>
<td>Pdx1-cre; Ink4A/Arf&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>no</td>
<td>no</td>
<td>--</td>
<td>Aguirre 2003</td>
</tr>
<tr>
<td>Pdx1-cre; LSL-KRAS&lt;sup&gt;G12D&lt;/sup&gt;, Ink4A/Arf&lt;sup&gt;−/−&lt;/sup&gt;; p53&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>mPanIN</td>
<td>yes</td>
<td>2 mo</td>
<td>Aguirre 2003, Bardeesy 2006a</td>
</tr>
<tr>
<td>Pdx1-cre; LSL-KRAS&lt;sup&gt;G12D&lt;/sup&gt;, Ink4A/Arf&lt;sup&gt;−/−&lt;/sup&gt;; p53&lt;sup&gt;−/−&lt;/sup&gt;; Smad&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>mPanIN</td>
<td>yes</td>
<td>1-2 mo</td>
<td>Bardeesy 2006a</td>
</tr>
<tr>
<td>Pdx1-cre; Smad&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>no</td>
<td>no</td>
<td>--</td>
<td>Bardeesy 2006b, Izeradjene 2007; Kojima 2007</td>
</tr>
<tr>
<td>Pdx1-cre; LSL-KRAS&lt;sup&gt;G12D&lt;/sup&gt;, Smad&lt;sup&gt;−/−&lt;/sup&gt;; Ink4A/Arf&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>mMCN</td>
<td>yes</td>
<td>9 mo</td>
<td>Izeradjene 2007</td>
</tr>
<tr>
<td>Pdx1/Ptf1a-cre; LSL-KRAS&lt;sup&gt;G12D&lt;/sup&gt;, Smad&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>mIPMN</td>
<td>yes</td>
<td>8 mo</td>
<td>Bardeesy 2006b; Kojima 2007</td>
</tr>
<tr>
<td>Ptf1a-cre; LSL-Tgfbr2</td>
<td>no</td>
<td>no</td>
<td>--</td>
<td>Ijichi 2006</td>
</tr>
<tr>
<td>Ptf1a-cre; LSL-KRAS&lt;sup&gt;G12D&lt;/sup&gt;, Tgfbr2</td>
<td>mPanIN</td>
<td>yes</td>
<td>2 mo</td>
<td>Ijichi 2006</td>
</tr>
<tr>
<td>Pdx1/Ptf1a-cre; LSL-KRAS&lt;sup&gt;G12D&lt;/sup&gt;, Smad&lt;sup&gt;−/−&lt;/sup&gt;; Ink4A/Arf&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>mIPMN</td>
<td>yes</td>
<td>4 mo</td>
<td>Bardeesy 2006b</td>
</tr>
</tbody>
</table>

IPMN, intraductal papillary mucinous neoplasia; MCN, mucinous cystic neoplasia; mo, month; PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma; yr, year. (Bardeesy 2006a [53], Bardeesy 2006b [54])

Pancreatic cancer is an adult-onset disease, thus mutant KRAS expression in the developing mouse pancreas may not faithfully represent PDAC pathogenesis. Conflicting
studies have been reported concerning the ability of oncogenic KRAS to transform adult mouse pancreas cells. Cellular damage from acute pancreatitis caused by caerulein treatment was found to be required to precede KRAS\textsuperscript{G12V} expression in acinar cells to facilitate adult-onset malignancy [55]. However, studies from other groups have reported that KRAS\textsuperscript{G12D} expression in adult acinar cells under the control of an ELA or p48 promoter develops mPanIN lesions identical to models where mutant KRAS is expressed in the developing mouse pancreas. Discordance between these studies may be the result of the mouse strain, and the system or promoter used to express oncogenic KRAS [52,56-58]. Together these studies demonstrate that oncogenic KRAS expression can initiate the transformation in differentiated adult pancreatic cells.

### 1.5.2 KRAS and p16\textsuperscript{INK4A} models

Targeted p16\textsuperscript{INK4A} loss in the developing mouse pancreas does not cause neoplastic lesions. Knocking out p16\textsuperscript{INK4A} in combination with KRAS\textsuperscript{G12D} expression decreases latency in mPanIN formation, invasive PDAC, and metastatic disease compared to KRAS\textsuperscript{G12D} expression alone. The rapid advancement of mPDAC results in death by 11 weeks of age. Histological examination of the tumours revealed well to poorly differentiated carcinoma reminiscent of human disease. Molecular analyses of the tumours isolated from the transgenic mice demonstrated functional p53 and robust Smad4 expression [59]. This model of pancreatic cancer suggests that p16\textsuperscript{INK4A} restricts mutant KRAS from transforming mouse pancreata into invasive carcinoma.
1.5.3 **KRAS and p53 models**

Targeted p53\textsuperscript{R175H} expression in pancreatic progenitor cells does not impair pancreatic development nor promote transformation of acinar cells. However, concomitant p53\textsuperscript{R175H} and KRAS\textsuperscript{G12D} expression promotes invasive PDAC, metastatic disease, and decreases median survival by 7 months. Tumour derived cell lines generated from the mouse models demonstrated uniform Smad4 and p16\textsuperscript{INK4A} expression. These cell lines were genically unstable and had a large number of anaphase bridges, abnormal mitotic figures, increased number of centrosomes, and large structural genomic changes [60].

Knocking out p53 in the mouse pancreatic progenitor cells in combination with KRAS\textsuperscript{G12D} expression accelerates the progression of lethal PDAC by 2 months of age. Utilisation of array-comparative genomic hybridization (aCGH) demonstrated genomic instability analogous to the findings of the p53\textsuperscript{R175H} and KRAS\textsuperscript{G12D} model [53,60]. Moreover, KRAS\textsuperscript{G12D};p53\textsuperscript{−/−};p16\textsuperscript{INK4A−/−} mouse models had significantly reduced tumour latency compared with KRAS\textsuperscript{G12D};p16\textsuperscript{INK4A−/−} mouse models [53]. These findings indicate that p53 functions as a more potent restriction point than p16\textsuperscript{INK4A}.

1.5.4 **KRAS and Smad4 models**

Smad4 plays a prominent role during gastrulation and mesoderm formation so its loss leads to early embryonic lethality, whereas Smad4\textsuperscript{−/−} mice do not develop any abnormalities [61,62]. Smad4 is dispensable for pancreatic organogenesis and its loss does not initiate pancreatic cancer [54,63,64]. Targeted KRAS\textsuperscript{G12D} expression and Smad4 deficiency in the developing mouse pancreas rapidly develops lesions resembling IPMNs and advanced mPanIN. Median survival was significantly reduced to three months. Tumours isolated from
KRAS<sub>G12D</sub>;Smad4<sup>−/−</sup> mice retain more epithelial markers than tumours with intact Smad4 [54]. Kojima and colleagues also generated a KRAS<sup>G12D</sup>;Smad4<sup>−/−</sup> mouse model that developed fibrotic neoplastic lesions with advanced mPanIN and IPMN appearance. The average survival for the transgenic mice was 4 months and immunohistochemical analysis of the tumours also revealed that the tumours retained epithelial markers such as CK19 positivity and mucin expression [64].

Izeradjene and co-authors reported that targeted KRAS<sup>G12D</sup> expression and Smad4 loss produces mMCN which advanced into invasive and metastatic mPDAC with a median survival of 8 months. Molecular analyses of these tumours revealed enhanced EGFR and Her2/neu expression, and p16<sup>INK4A</sup> loss through mutation and promoter methylation. Fewer centrosome amplifications and translocations were observed compared to tumours isolated from KRAS<sup>G12D</sup>;p53<sup>−/−</sup> mice indicating that Smad4 has a lesser role in protecting genomic integrity than p53 [63]. However, concomitant p16<sup>INK4A</sup> and Smad4 loss in combination with mutant KRAS expression in the mouse pancreas led to the rapid onset of mPDAC with a latency in tumour formation that was similar to KRAS<sup>G12D</sup>;p16<sup>INK4A/−</sup> mice [54]. These data indicate that Smad4 and p16<sup>INK4A</sup> regulate distinct pathways in suppressing PDAC progression.

In a small subset of PDAC cases TGFBR1 and TGFBR2 are altered [65]. Ijichi and colleagues targeted KRAS<sup>G12D</sup> expression in combination with TGFBR2 loss. This model had disease progression and histopathology similar to human PDAC, and a median survival of 59 days [66]. The findings of these studies indicate that disrupting the TGF-β signalling pathway and activating the KRAS signalling pathway contribute synergistically to the development of PDAC.

MCNs and IPMNs are cystic precursors that have potential to advance to invasive PDAC. MCNs and IPMNs are localised in different regions in the pancreas and are histopathologically distinct. MCNs are localised in the body and tail of the pancreas and do not involve the duct
system. MCNs have been reported to harbour activating mutations in KRAS as well as mutations in p53 and Smad4. IPMNs are found within the main or major branch pancreatic duct. Activating KRAS mutations occur less frequently in IPMN than in PanINs, and a quarter of these patients harbour LKB1 mutations. Smad4 is usually intact in non-invasive IPMNs, but can be lost during metastasis. [67]. The development of cystic lesions in these mouse models represents an alternate route to developing mPDAC, and is in contrast to other transgenic models where targeted KRAS\textsuperscript{G12D} expression is associated with mPanIN formation. Temporally, these models may also suggest that Smad4 loss needs to follow KRAS activation, and/or p53 or p16\textsuperscript{INK4A} inactivation in order to recapitulate the PanIN to PDAC repertoire [54,63].

1.6 Human pancreatic duct epithelial cell line

The majority of pancreatic cancers occur within the duct. The establishment of an immortalized epithelial cell line from human pancreatic duct provides an appropriate \textit{in vitro} model to examine the dynamics of pancreatic duct cell carcinogenesis. Pancreatic epithelial cell models such as the immortalised Nestin positive (HPNE) cells derived from the adult endocrine pancreas [68], and non-human pancreatic duct epithelial cell lines from sources such as hamster [69], rat [70], mouse [71], and bovine [72] have also been established.

The human pancreatic duct epithelial cell line (HPDE) was established from an explant of pancreatic duct. Primary culture of the pancreatic duct led to epithelial cell expansion. Immortalisation is required for continuous culture since normal epithelial cells have limited replicative ability. Immortalization of the HPDE cell line was achieved by infection with the amphotropic retrovirus, LXSN16E6E7, containing the E6 and E7 genes of HPV-16 [73]. The
products of the E6 and E7 genes target p53 and Rb, respectively. Single clones were isolated from the HPDE cell line, and two clones HPDE6-E6E7c7 and HPDE6-E6E7c11 were found to be paraploid and were further characterised. Both clones were cultured for up to 35 passages, demonstrated telomerase activity, and displayed a similar phenotype to the primary culture. aCGH studies performed on the two clones revealed chromosomal losses on 3p, 10p12, 13q14 and a gain of chromosome 20. There was a further loss of chromosome 22 in clone 11. Thus, HPDE6-E6E7c7 (H6c7) was used for subsequent studies [74].

Molecular analysis of the H6c7 cell line demonstrated wildtype KRAS expression. In comparison to other pancreatic cancer cell lines, H6c7 exhibited low mRNA expression of EGFR, MET, and FGF7 [73,75]. γ-irradiation failed to induce p53 expression due to E6 expression. p16^{INK4A} mRNA levels were markedly enhanced in comparison with the primary pancreatic duct culture due to E7 expression. The H6c7 cell line expresses Smad4, and responds to the cytostatic effects of TGF-β stimulation by upregulating p21 and p27. The H6c7 cell line expresses duct specific markers carbonic anhydrase II, Mucin-1, and cytokeratins 8, 18, and 19 [74,75]. The H6c7 cell line is anchorage dependent and fails to form colonies in soft agar. Implantation of the H6c7 cell line into the neck fat pad of severe combined immunodeficient (SCID) mice fail to form tumours [74]. Despite the changes in karyotype and the loss of p53 expression, the H6c7 cell line represents a near normal human model that can be utilised to study the effects of genetic alterations common to PDAC.

1.7 Human PDAC cell lines

The difficulty in obtaining fresh human tumour tissue and the maintenance of animal models makes employing human PDAC cell lines a particularly attractive research tool to examine the contributions of genetic aberrations. These cells lines were established from donor
patients between 26 and 65 years of age either from the primary tumour or from metastases [76].

Genetic analysis and xenotransplantation studies have shown that many of the cell lines retain the genetic lesions and histological differentiation consistent with the original tumour [77,78].

The four most common mutations that occur in PDAC are found in similar percentages across these lines. The most widely used pancreatic cancer cell lines are listed in the table below.

### Table 1.2 Genetic aberrations in commonly used pancreatic cancer cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>Differentiation</th>
<th>KRAS</th>
<th>p53</th>
<th>p16</th>
<th>SMAD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsPC1</td>
<td>Ascites</td>
<td>P</td>
<td>G12D</td>
<td>C135FS</td>
<td>WT</td>
<td>LOH/R100T</td>
</tr>
<tr>
<td>BxPC3</td>
<td>Pancreas</td>
<td>M</td>
<td>WT</td>
<td>Y220C</td>
<td>WT/Meth</td>
<td>HD</td>
</tr>
<tr>
<td>CaPan-1</td>
<td>Liver</td>
<td>W</td>
<td>G12V</td>
<td>A159V</td>
<td>HD</td>
<td>LOH/F577L</td>
</tr>
<tr>
<td>CaPan-2</td>
<td>Liver</td>
<td>W</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>CFPAC</td>
<td>Liver</td>
<td>W</td>
<td>G12V</td>
<td>C242R</td>
<td>WT/Meth.</td>
<td>HD</td>
</tr>
<tr>
<td>Colo357</td>
<td>Lymph node</td>
<td>M</td>
<td>G12D</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>HPAC</td>
<td>Pancreas</td>
<td>M to W</td>
<td>G12D</td>
<td>WT</td>
<td>E121*</td>
<td>WT</td>
</tr>
<tr>
<td>HPAF II</td>
<td>Ascites</td>
<td>W</td>
<td>G12D</td>
<td>P151S</td>
<td>20-25 Del</td>
<td>WT</td>
</tr>
<tr>
<td>Hs766T</td>
<td>Lymph node</td>
<td>P</td>
<td>G12D</td>
<td>Ex. 2-4Del</td>
<td>In. 2 splice</td>
<td>HD</td>
</tr>
<tr>
<td>Mia PaCa2</td>
<td>Pancreas</td>
<td>P</td>
<td>G12C</td>
<td>R248W</td>
<td>HD</td>
<td>HD</td>
</tr>
<tr>
<td>PANC1</td>
<td>Pancreas</td>
<td>P</td>
<td>G12D</td>
<td>R273H</td>
<td>HD</td>
<td>WT</td>
</tr>
<tr>
<td>PK1</td>
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<td>G12D</td>
<td>M237I</td>
<td>HD</td>
<td>HD</td>
</tr>
<tr>
<td>Su86.86</td>
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<td>M</td>
<td>G12D</td>
<td>G245S</td>
<td>HD</td>
<td>WT</td>
</tr>
<tr>
<td>Sw 1990</td>
<td>Spleen</td>
<td>M to W</td>
<td>G12D</td>
<td>WT</td>
<td>HD</td>
<td>WT</td>
</tr>
</tbody>
</table>

The cell lines were derived from tumour sites as noted in origin and differentiation refers to histology of the xenograft. *, nonsense mutation; Ex., exon; Del, deletion; HD, homozygous deletion; In., intron; LOH, Loss of heterozygosity; M, moderate; Meth, methylated; P, poor; W, well differentiated; WT, wildtype; [76-79,79].

### 1.8 RAS-induced transformation

During malignant transformation, cells acquire genetic alterations that can override homeostatic mechanisms which control phenotypes such as proliferation, survival, and motility. The majority of studies examining transformation have used murine models. Though these
studies have yielded insights into the mechanisms involved in human cancers, differences still exist in the proficiency of human and rodent cells to transform. Telomere length has been regarded to be one of the key differences between rodent and human cells. Rodent cells have longer telomeres than human cells, and are more efficiently transformed since immortalisation does not represent a barrier to tumorigenicity [80]. Moreover, it has been observed that human cells require additional genetic changes beyond what is required for tumorigenic transformation of rodent cells [81,82].

The role of oncogenes in tumourigenesis have extensively studied mutant RAS due to its frequency in human epithelial malignancies. Experimental mouse models and cell culture studies have demonstrated the ability of mutant RAS to promote tumourigenesis. Activating point mutations in RAS can constitutively activate downstream effector pathways involved in survival, motility, and proliferation [16]. However, oncogenic RAS expression results in cell cycle arrest, or oncogene induced senescence. Oncogene induced senescence can be bypassed through the loss of p53 and p16 [83]. Thus, immortalisation methods that have utilized E6 and E7, or SV40 which target p53 and RB can permit RAS induced transformation [80].

1.8.1 Transformation of HPDE cells with KRAS\textsuperscript{G12V}

KRAS mutations occur early on in pancreatic duct carcinogenesis and are frequently found in PDAC [12]. Stable KRAS\textsuperscript{G12V} expression was introduced in H6c7 cells by a retroviral vector using an ecotropic packaging system (eco-KRAS\textsuperscript{G12V}). The eco-KRAS\textsuperscript{G12V} cell line maintained an epithelial phenotype and TGF-β sensitivity. KRAS\textsuperscript{G12V} expression did not alter proliferation rates or cause anchorage-independent growth. Oncogenic KRAS triggered activation of AKT and MAPK. Subcutaneous and orthotopic implantation of the eco-KRAS\textsuperscript{G12V}
cell line generated tumours in half of the SCID mice assessed. Histological analyses of the
tumours revealed poorly differentiated carcinoma. Metastases were not observed in mice
orthotopically implanted with the eco-KRAS\textsuperscript{G12V} cell line. These data indicate that mutant KRAS
can initiate transformation of the H6c7 cell line [84].

Microarray profiling performed on the eco-KRAS\textsuperscript{G12V} cell line identified transcriptional
changes induced by KRAS expression. 584 genes were found to be upregulated and 465 were
downregulated in comparison to H6c7 cells. The upregulated genes were compared to a
compiled list of publicly available microarray and serial analysis of gene expression data on
genes that are differentially expressed in pancreatic cancer. From this comparison, 42 genes that
were previously reported to be overexpressed in pancreatic cancer were also identified in the
eco-KRAS\textsuperscript{G12V} cell line. Thus, this \textit{in vitro} model can serve as a powerful tool for determining
potential biomarkers for early detection of PDAC [84].

1.8.2 \textit{Transformation of HPNE cells with KRAS\textsuperscript{G12D}}

The Nestin expressing HPNE cell line represents an alternative normal pancreatic cell
line of human origin. Nestin is a neuronal stem cell marker that is expressed in the developing
pancreas, and in adults it is expressed in the pancreatic islets and ducts [85]. The HPNE cell line
was immortalised through the expression of hTERT, and expresses wildtype KRAS, p16\textsuperscript{INK4A},
and p53. However, it fails to express duct specific markers carbonic anhydrase II and CK19
[68]. The E6 and E7 genes, and small t (st) antigen from SV40 (HPNE-E6/E7/st) were utilised to
enhance KRAS\textsuperscript{G12D} transforming ability. The E6 and E7 genes were employed to target the p53
and p16 genes, respectively, and the small t antigen was used to transform the HPNE cell line
[86]. Mutant KRAS expression in the HPNE-E6/E7/st cell line increased proliferation,
decreased contact inhibition, promoted anchorage-independent growth, activated downstream MAPK and AKT effector pathways, stimulated migration and invasion, and led to tumour formation in nude mice [87].

1.8.3 Transformation of bovine pancreatic duct cells with KRAS<sup>G12V</sup>

The pancreatic duct epithelial cell line (PDEC) was isolated from explants of bovine pancreatic duct. The PDEC cell line was immortalised using SV40 large T antigen which targets both p53 and pRB. The PDEC cell line expresses ductal markers carbonic anhydrase II, CK19, and Mucin-1 [72,88]. Stable KRAS<sup>G12V</sup> expression promoted transcription of cell cycle inhibitors p21 and p27, anti-apoptotic genes BCL-2 and BCL-xL, and increased MAPK activation. Furthermore, KRAS expressing PDEC cells were unable to grow in an anchorage independent manner, but were able to generate tumour formation [72].

1.8.4 Transformation of rodent pancreatic duct cells with KRAS<sup>G12V</sup>

Rodent pancreatic duct epithelial cells (rPDECs) were isolated from serial digestion of rat or mouse pancreatic ducts. rPDECs undergo senescence in culture, however forced KRAS<sup>G12V</sup> expression by microinjection bypasses cell cycle arrest [70]. Microinjection of KRAS<sup>G12V</sup> increased cell size which was mediated by activation of the PI3K pathway, and promoted the loss of E-cadherin expression and gain of N-cadherin [70]. Induction of senescence in rPDECs is dependent on the presence of p16<sup>INK4A</sup> [71]. Oncogenic KRAS expression upregulates Twist which bypasses senescence by inhibiting p16<sup>INK4A</sup> transcription [71].
1.9 The interaction between the TGF-β and KRAS signalling pathways

TGF-β exerts growth inhibition through cell cycle arrest in epithelial cells. Disruptions in the canonical signaling pathway can transition TGF-β from tumour suppressor to tumour promoter. This shift in TGF-β function enhances tumour growth and invasion. Smad4 null pancreatic cancer cell lines, such as BxPC3 and CFPAC-1, are refractory to the cytostatic effects of TGF-β [89]. Despite Smad4 absence, TGF-β has been demonstrated to increase cell motility and invasion in the BxPC3 cell line [89,90]. TGF-β induced cell cycle arrest has been reported in Smad4 deficient pancreatic cancer cell lines, Hs766T and Colo357, which suggests that Smad-independent signalling exists. Although Smad proteins are considered important mediators for canonical signaling, Smad-independent TGF-β signalling has been implicated to involve RAS/Raf/MAPK signalling pathway through the presence of a functional TGF-β receptor complex. MAPK signalling has been implicated in regulating the expression of TGF-β responsive genes such as fibronectin and p21 [91-94]. MEK1 inhibitor PD98059 has been demonstrated to abrogate TGF-β-mediated cell cycle arrest in Hs766T and Colo357 cell lines [95]. The MiaPaCa-2 cell line expresses low levels of TGFB1 which fails to activate MAPK following TGF-β treatment [96]. Besides MAPK, there has been increasing evidence that TGF-β can also signal non-canonically through p38, JNK, RhoA, PI3K, Pak2, and Par6 [22,90,92,97]. These studies suggest that Smad4 loss can promote TGF-β mediated tumourigenesis through loss of cell cycle regulation and apoptosis.
Figure 1.4. *Crosstalk between the TGF-β and RAS signalling pathways.* The TGF-β signaling pathway can signal through canonical Smad or through non-Smad signaling pathways. The binding of the TGF-β ligand to the receptor complex initiates signaling through various effectors including the MAPK, JNK, p38, and RhoA (adapted from [22,90,92,97]).

Activating KRAS mutations are nearly ubiquitous in PDAC, however oncogenic KRAS has been reported to repress TGF-β signalling. Massague and colleagues have demonstrated that RAS attenuates Smad signalling through MAPK activation. MAPK phosphorylation at specific sites on the linker region between the MH domains in Smad2 and Smad3 can abrogate nuclear translocation [98,99]. MAPK activation can also lead to early proteasomal degradation of Smad4 [100]. In contrast, constitutively active Raf or KRAS$^{G12V}$ expression in mammary epithelial cells
has no effect on Smad2/3/4 translocation into the nucleus, DNA binding ability, or transcriptional activity after TGF-β stimulation. Rather, Raf activation inhibits cells from undergoing TGF-β-mediated apoptosis [101]. Thus, the RAS pathway may enhance the tumour promoting effects of TGF-β by disabling R-Smad function, however this may be cell-type specific.

TGF-β induced EMT in pancreatic cancer has been shown to be mediated through the PI3K signalling pathway. Phosphorylation of α- and β-catenin by PI3K enhances migration through reduced cell-cell adhesion [90]. TGF-β has been reported to stimulate actin cytoskeleton reorganization during EMT through the activation of RhoA and ROCK [102]. In breast cancer it has been reported that TGF-β can act on tight junctions through Par6 which promotes the phenotypic events associated with EMT [97]. These data indicate that the interaction between the TGF-β and other effector pathways associated with EMT are complex and can occur at multiple levels.

Targeted KRAS$^{G12D}$ expression coupled with SMAD4 loss in the developing mouse pancreas rapidly generates cystic lesions and mPanIN lesions compared to expression of KRAS$^{G12D}$ alone [54,63,64]. In the HPNE cell line, KRAS$^{G12D}$ expression and Smad4 knockdown enhances invasive ability by upregulating EGFR expression [103]. Altogether, these data indicate that disrupting the TGF-β signalling pathway and KRAS activation contribute synergistically to PDAC development.
1.10 Smad4 as a tumour suppressor gene

The prevalence of 18q loss in pancreatic cancer indicates the importance of Smad4 loss in the evolution of pancreatic carcinogenesis [45]. Smad4 absence is associated with poorer survival and widespread metastasis [104]. Smad4 is dispensable for the tumour-promoting effects of TGF-β, however its presence is critical for mediating the cytostatic effects of the TGF-β signalling pathway [36]. Transferring chromosome 18 into PDAC cell lines BxPC3, MiaPaCa2, and PANC1 decreased in vitro proliferation, anchorage independent growth, invasion, and in vivo tumour growth. Restoring Smad4 and additional tumour suppressor genes that reside on chromosome 18 can repress tumorigenicity of pancreatic cancer cell lines [105].

The consequences of Smad4 expression on tumorigenicity of PDAC has been examined in numerous cell line models. Reconstituting Smad4 in the Smad4 null Hs766T cell line did not fully restore TGF-β responsiveness due to decreased TGFB1 levels. Smad4 diminished Hs766T tumorigenicity by reducing vascularity through repression of VEGF expression and upregulation of angiogenesis inhibitor thrombospondin 1 [106]. Smad4 restoration in the Smad4 null pancreatic cancer cell lines, BxPC3 and Capan-1, reinstates TGF-β sensitivity, and promotes p15 and p21 expression thereby reducing in vivo tumour growth [107-110]. Studies performed in colorectal, breast, and ovarian cancer cell models have reported analogous findings of decreased tumour growth and reversion of cells to a more epithelial-like state [106,109,111,112]. These data illustrate the importance of Smad4 as a tumour suppressor by mediating the cytostatic effects of TGF-β.
1.11 **Lipocalin 2 (LCN2)**

LCN2 is also known as neutrophil gelatinase-associated lipocalin (NGAL) and belongs to a diverse family of lipocalins [113]. LCN2 has been detected as a monomer, homodimer, and heterodimer with MMP-9 [114]. LCN2 protein consists of eight β-sheets that compose a barrel shaped structure which forms a cavity that permits hydrophobic ligand binding, facilitates iron delivery into cells, and binds to soluble extracellular macromolecules [114,115]. LCN2 knockout mice have increased susceptibility to bacterial infections as its iron binding role acts as a potent bacteriostatic agent [116,117]. LCN2 has been identified as an early marker for acute kidney injury, and inflammatory conditions affecting the bowel and respiratory system [113]. Increased LCN2 expression has been observed in a number of cancer types including breast, lung, ovary, thyroid, esophageal, and pancreas [118-122]. Elevated LCN2 expression has been hypothesized to promote tumourigenicity.

LCN2 was first discovered in neutrophils bound to the gelatinase, MMP-9. The disulfide linked LCN2 dimer has been identified to enhance MMP-9 activity by binding to the N-terminus of MMP-9 after its been cleaved and prolongs its activity by protecting MMP-9 from autodegradation [123,124]. This LCN2-MMP-9 complex has been associated with enhanced invasion and increased metastatic potential in breast cancer [122,125,126], and greater tumour invasion in esophageal and gastric cancers [121,127]. Besides its role in invasion, recent studies have demonstrated LCN2 promotes cell migration which enhances gastrointestinal mucosal regeneration [128]. Furthermore, forced LCN2 expression decreases E-Cadherin, and increases vimentin and fibronectin expression which enhances invasion and migration in the breast cancer cell line, MCF-7 [129]. Together, these studies indicate that LCN2 promotes tumour invasion by enhancing MMP-9 activity and enhances migration through promotion of EMT.
Evasion of apoptosis is a hallmark of cancer cells. Several recent studies have revealed that LCN2 may also be an anti-apoptotic protein. Stable LCN2 expression in A549 and MCF-7 cell lines reduced sensitivity to the pro-apoptotic phosphoinositide-dependent kinase 1 (PDK1) inhibitor [130]. The iron binding ability of LCN2 attenuates apoptosis by downregulating Bim expression and caspase-9 activation in the thyroid carcinoma cell line FRO [120]. LCN2 facilitates cortical tubules recovery after ischemia-reperfusion injury [131]. Thus, this pro-survival function of LCN2 can further promote tumorigenicity.

Despite the work that has illustrated the multi-faceted role that LCN2 plays in cancer, several studies have shown disparate findings. In HRAS transformed breast cancer cells, LCN2 expression diminishes EMT, tumour growth, and invasion by attenuating Raf activation [132]. LCN2 expression in the colon cancer cell line, KM12SM, suppresses tumour growth and metastasis [133]. Recent work in pancreatic cancer has shown that elevated LCN2 expression attenuates adhesion through inhibition of focal adhesion kinase (FAK) and reduction of angiogenic potential resulting in decreased tumour growth [134]. In leukemic mouse models LCN2 secreted from BCR-ABL positive cells induces apoptosis by depleting iron in normal hematopoetic cells [135]. These contradictory findings suggest that the role that LCN2 plays in cancer may be context dependent and cell type specific.

1.12 Hypothesis

Introduction of multiple genetic aberrations into a near normal human pancreatic duct cell line, HPDE, can recapitulate the multi-step molecular basis of pancreatic duct cell carcinogenesis. Smad4 inactivation can cooperate with KRAS
textsuperscript
G12V oncogene to transform HPDE cells. Furthermore, oncogenic KRAS can induce expression of novel genes which may promote tumourigenicity in pancreatic cancer cells.
1.13 Specific Aims

1. KRAS$^{G12V}$ expression in the HPDE cell line induced expression of several genes that were also found to be overexpressed in PDAC. One of these genes was LCN2, which is emerging as a new biomarker in several cancer types. The goals of this study were to elucidate its function and examine its expression in PDAC progression. The findings from this work are presented in Chapter 2 and were published in the journal PLOSOne.

2. The frequent inactivation of Smad4 indicates its importance in malignant progression of PDAC. shRNA was employed to address the consequences of Smad4 loss in the HPDE model. Furthermore, KRAS$^{G12V}$ was expressed to determine if this would synergise with Smad4 depletion to mediate transformation of the HPDE cell line. The results of this study are presented in Chapter 3.

3. Smad4 was incompletely suppressed utilising shRNA and retained blunted TGF-β sensitivity. A TGF-β insensitive HPDE cell line was employed to examine the consequences of Smad4 deficiency and if KRAS$^{G12V}$ expression could mediate malignant transformation of the HPDE cell line. The results of this study are presented in Chapter 4, and are in preparation for submission in conjunction with the results of Chapter 3.
Chapter 2

**Lipocalin2 promotes invasion, tumourigenicity and gemcitabine resistance in pancreatic ductal adenocarcinoma**

Lisa Leung, Nikolina Radulovich, Chang-Qi Zhu, Shawna Organ, Bizhan Bandarchi, Melania Pintilie, Christine To, Devang Panchal, and Ming-Sound Tsao.

A similar report was published here as follows:


Author contributions (percentage of contribution):

LL: conceived the study, conducted the *in vitro* and *in vivo* experiments, analysed the tissue microarray, performed the statistical analysis, and wrote the manuscript (86%)

NR: assisted in the cloning of the LCN2 expression construct and cell line generation (5%)

CZ, CT, and SO: analysed microarray data (5%)

BB: assessed blood vessel density and tissue microarray (2%)

MP: analysed tumour growth curves (1%)

DP: aided in toxicity studies (1%)
2.1 Abstract

Lipocalin 2 (LCN2) is a small secreted protein and its elevated expression has been observed in several cancer types. LCN2 has been reported to promote resistance to drug-induced apoptosis, enhance invasion through its physical association with matrix metalloproteinase-9, and promote *in vivo* tumor growth. LCN2 was commonly expressed in primary pancreatic ductal adenocarcinoma (PDAC) tissue and in Pancreatic Intraepithelial Neoplasia (PanIN) lesions. LCN2 immunohistochemical staining levels increased significantly in high-grade precursor lesions. Downregulation of LCN2 in two pancreatic ductal adenocarcinoma cell lines (BxPC3 and HPAF-II) with high LCN2 expression significantly reduced attachment, invasion, and tumour growth *in vivo*, but not proliferation or motility. In contrast, LCN2 overexpression in PANC1, with low endogenous expression, significantly increased invasion and attachment *in vitro*, and enhanced tumor growth. Suppression of LCN2 in BxPC3 and HPAF-II cells increased their sensitivity to gemcitabine *in vitro*, and *in vivo* when BxPC3 was tested. Expression microarray analysis revealed that LCN2 upregulated the expression of genes involved in survival and attachment. Furthermore, LCN2 increased expression of VEGF and HIF1A, which contribute to enhanced vascularity. Overall, these results demonstrate that LCN2 plays an important role in the malignant progression of PDAC, and may be a therapeutic target for this disease.
2.2 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in North America with an overall 5-year survival rate of <5% [136]. Previous PDAC microarray studies have revealed novel genes associated with disease progression. One of these was lipocalin-2 (LCN2), which was significantly overexpressed in PDAC cell lines and primary tumors compared to normal pancreas [104,137]. LCN2 expression was also enhanced following KRAS oncogene expression in the normal human pancreatic duct epithelial cell line H6c7 [84].

LCN2 is also known as neutrophil gelatinase-associated lipocalin (NGAL) and belongs to a diverse family of lipocalins [113]. It binds covalently and non-covalently with a wide range of macromolecules including small hydrophobic ligands, soluble extracellular macromolecules, and iron [138]. Its expression is upregulated in epithelial cells under inflammatory conditions including appendicitis, organ damage, and pancreatitis [113,139]. Overexpression of LCN2 has also been observed in a number of cancer types including breast, lung, ovarian, thyroid, esophageal, and PDAC [118-122]. However, the precise role of LCN2 in cancer has not been completely defined. The covalent complex of LCN2 and MMP-9 has been associated with enhancing invasion and metastasis in breast cancer [122,125,126], poorer clinical outcome and improved migration in gastric cancer, [127,128], and increased depth of tumour invasion in esophageal cancer [121]. In addition to its role in regulating MMP-9 activity, LCN2 has also been shown to promote cell survival in A549 and MCF-7 cells when treated with phosphoinositide-dependent kinase 1 (PDK1) inhibitors [130]. Its function in iron binding and transport has recently been shown to block the induction of the pro-apoptotic protein Bim and activation of caspase-9 which attenuates apoptosis [120]. The function of LCN2 in PDAC
remains unclear. In this study, we examined the expression of LCN2 in PDAC precursor lesions of various grades and in PDAC tissue samples. We also utilised tissue culture and mouse xenograft models to examine the function of LCN2 in PDAC. Here, we report that LCN2 contributes to the invasive, angiogenic, and drug resistant phenotypes in pancreatic cancer.

2.3 Materials and Methods

Cell culture and in vitro assays: Human PDAC cell lines, BxPC3, HPAF-II and PANC1 were obtained from the American Type Culture Collection (Manassas, VA). BxPC3 was cultured in RPMI media supplemented with 10% FBS. HPAF-II and PANC1 cells were cultured in DMEM media supplemented with 10% FBS. H6c7, H6c7 KRASG12V, and H6c7KrT cell lines were generated as previously described [84].

Invasion Assay. Fifty thousand cells were seeded onto a 24-well transwell cell culture plates (BD Biosciences, Mississauga, Ontario) fitted with multiporous (8 µm pore size) polycarbonate membranes (Falcon, Mississauga, Ontario) for the invasion assay. The upper chambers of the membrane were coated with 13 µg matrigel or 7.5 µg of collagen IV, and the lower chambers were filled with media supplemented with 5 µg/ml fibronectin. After 48 hours, cells were fixed with 0.5% glutaraldehyde and stained with 0.5% crystal violet reagent for 48 hours. The upper chamber was washed gently with distilled water and cotton swabs were used to remove the cells from the upper surface of the membrane, leaving the migratory cells on the underside the membrane. The membrane insert was left to dry overnight. The membrane was removed and mounted onto glass slides for scanning. Whole slides were scanned using the Aperio Scanscope XT (Aperio Technologies Inc, Vista, California). Migratory cells were counted in six
independent fields using Aperio Imagescope software (Aperio Technologies Inc, Vista, California).

**Adhesion assay.** 100 000 (BxPC3, HPAF-II, and PANC1) or 250 000 cells (H6c7KrT) cells were seeded onto a 24-well dish coated with fibronectin and collagen (Sigma Aldrich, Mississauga, Canada) for 30 minutes (BxPC3, HPAF-II, and PANC1) or 45 minutes (H6c7KrT). The plates were then washed thrice with PBS, fixed with 96% ethanol, stained with 0.2% crystal violet, and lysed with 0.1% Triton X-100. The resulting lysate was read at 590 nm on a Tecan XFlour4 plate reader (Tecan, Mannedorf, Switzerland).

**Propidium iodide (PI; Sigma Aldrich) exclusion assay.** 2.5x10⁵ cells were seeded in 6-well dishes and treated with 10µM gemcitabine for 72 hours. PBS was used as a control. Live cells were stained in PBS supplemented with 0.5% BSA and 1 µg/ml PI, and were analyzed by flow cytometry using the BD FACScan (BD, Mississauga, Canada).

**Half maximal inhibitory concentration (IC50).** Cells were grown in increasing concentrations of gemcitabine at 0, 0.001, 0.01, 0.1, 1, 5, 10, 20. 50, and 100 µM for 48 hours. Cell viability was assessed by MTS assay (Promega, Madison, WI).

**Wound healing assay.** 2.5x10⁵ cells were seeded on 6-well dishes. Upon confluence cells were serum starved and scratched. Images were taken at 0, 24, and 48 hours to assess motility.

**Quantitative PCR.** Total RNA was isolated from cells and PCR was performed as described before [140]. The assays were performed using a Stratagene MX3000P (Stratagene, La Jolla,
California). The relative quantification of gene expression was determined using the comparative C<sub>T</sub> method. The values of ribosomal protein S13 (RPS13), TATA box binding protein (TBP), and β-actin (ACTB) gene expression were used to normalize the expression data.

Individual primer sets are listed in the table below.

**Table 2.1 QPCR primer sequences**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>RPS13</td>
<td>ggctgtttcataagcatcttg</td>
<td>aatacagagccacaggtgaa</td>
</tr>
<tr>
<td>TBP</td>
<td>ggccattatgtcactgaga</td>
<td>tagcagcaggtatgagacat</td>
</tr>
<tr>
<td>ACTB</td>
<td>tctaaagcaacccactctct</td>
<td>gggagagactgaggccat</td>
</tr>
<tr>
<td>LCN2</td>
<td>gggtttcacaagttttctcaa</td>
<td>tccgaagacgtctctgttt</td>
</tr>
<tr>
<td>AIFM</td>
<td>gaagagccaggaagtagtga</td>
<td>gc gagatcacaagttgt</td>
</tr>
<tr>
<td>BIRC2</td>
<td>ttttccaggtctctgtctct</td>
<td>ccaatgtgacagatgtcct</td>
</tr>
<tr>
<td>FAIM</td>
<td>tgccttaagtgacggagtcc</td>
<td>gtctttgagcgctccaact</td>
</tr>
<tr>
<td>MCL-1</td>
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<td>tagcagcagcagctcact</td>
</tr>
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<td>agtaaccaggtgctttgatt</td>
</tr>
</tbody>
</table>

**LCN2 mRNA silencing and overexpression.** LCN2 expression was stably downregulated by shRNA retroviral transduction method. The shRNA sequences were ligated into the pSUPER GFP retrovirus vector after linearization with BglII and HindIII (New England Biolabs, Pickering, Canada). The shRNA oligonucleotides used were: LCN2KD1: CGCATGCTATGGTGTCTTCAA, LCN2KD2: AACCTCCGTCTCTTATTAGAAA, LCN2KD3: GAGTTCACGCTGGCAACATTA, and non-silencing control siRNA sequence: TTCTCCGAACGTGTCACGT (Qiagen, Dusseldorf, Germany). The shRNA retroviral expression vectors were generated using Phoenix-amphotropic packaging cell line (ATCC). The LCN2 expression construct was generated using our modified Gateway recombination cloning system [141]. LCN2 cDNA was reversed transcribed from the BxPC3 mRNA and amplified...
using the Platinum Pfx DNA polymerase (Invitrogen, Burlington, Canada) and gene specific primers (F: 5’CTGCCGCACCAGCCCCGAAAGGCGCGCCT3’; R: 3’GACGGCGTGTCGGGCTTTCCGGCGCAGGGA5’). The amplicon was subcloned into an entry vector pENTR-CMVON plasmid after linearization with Ascl and SwaI restriction enzymes (New England Biolabs), which then underwent recombination with a lentiviral plko.YFP destination vector using LR Clonase II (Invitrogen). The resulting plko.YFP-LCN2 vector was stably transduced by lentiviruses.

**Immunoblot and gelatin zymography analysis.** Immunoblotting was performed as described previously [140]. The primary antibodies used include total and cleaved caspase-3 (Asp175), and GAPDH were purchased from Cell Signaling. *LCN2* was purchased from R&D Systems (AF1757; Minneapolis, MN). Visualization was accomplished by using HRP-linked anti-rabbit, anti-mouse (Cell Signaling, Danvers, MA), and anti-goat secondary antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Visualization was achieved by the ECL-Plus detection kit (GE Healthcare, Baie d’Urfe, Canada) on a Typhoon phospho- and fluorescent imaging system 9400 (GE Healthcare). Gelatin zymography was performed on conditioned media collected from H6c7KRT, BxPC3, HPAF-II, and PANC1, and protein extracts from xenografts as previously described [142]. Resulting blots and gels were analyzed with ImageJ software (NIH, Bethesda, MA).

**Animal work.** All studies were conducted using protocols approved by the Ontario Cancer Institute Animal Care Committee (animal use protocol 745.09). Tumor growth and implantation was assessed as described before [140]. To assess sensitivity to gemcitabine treatment, each cell line was implanted subcutaneously into 20 mice. Once the tumors reached a mean tumor
diameter of 5 mm, the mice were randomized into two study arms, based on tumor volume and body weight. Ten mice were treated with vehicle (PBS) and ten were treated with gemcitabine (100 mg/kg) every 7 days. Animals were sacrificed if tumors reached 15 mm or severe drug side effects were evident.

**Immunohistochemistry.** The construction of the PanIN and PDAC tissue microarrays was reported previously using a protocol approved by the Research Ethics Board of the University Health Network [84]. Immunohistochemistry was performed based on previously described methods [142]. LCN2 antibody (R&D Systems) was used at 1:2000 dilution following pepsin digestion. The relative LCN2 staining pattern and intensity were scored on a scale from 0 to 3 and multiplied by the percentage of positive cells which were scored as follows: 1:1-25%; 2:26-50%; 3:51-75%; 4:76-100%. Thus, the range of scores was between 0-12. The slides were scored independently by LL and BB, a board-certified pathologist. Blood vessels were counted as described before [142]. Briefly, blood vessels were identified by immunostaining with murine CD31 antibody. Blood vessels were scanned under 40x and 100x magnification, and were counted for discrete vessel formation. The number of discrete vessels found within a 100x field was counted five times for each xenograft assessed.

**Microarray analysis.** The effect of LCN2 suppression was evaluated by transcriptional profiling in the BxPC3 cell lines and xenografts using the Illumina HumanHT-12 v4 array (Illumina, San Diego, CA). The data were normalized using log2-transformation and quantile normalization. Moderated paired t-tests were used to compare samples and controls. Common differences in fold changes that were ≥ 1.5-fold were included in our analyses carried out using SAS v9.2. GO
term analysis of genes significantly associated with \textit{LCN2} using microarray analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (\textit{DAVID}) v6.7.

\textbf{Statistical analysis.} LCN2 immunostaining of tissue microarrays were analysed using the Kruskal-Wallis one-way ANOVA, data as indicated were analyzed using ANOVA, Student t-test, and linear regression for tumour growth comparison using GraphPad Prism 5 (La Jolla, CA). Data are presented as the means ± SEM. P values <0.05 were considered significant. The tumor volume measured over time was analyzed after log-transformation using the mixed effect regression model using the LCN2 (KD vs. NS) and the treatment (Gem vs. PBS) as explanatory variables using R 2.9.1 software.

2.4 \textbf{Results}

2.4.1 \textit{LCN2 expression in multi-stage pancreatic duct cell carcinogenesis.}

To determine the association between LCN2 immunoreactivity and PDAC pathogenesis, LCN2 expression was assessed by immunohistochemistry using a tissue microarray constructed to include normal (n=31), pancreatic intraepithelial neoplasia (PanIN)-1 (n=22), PanIN-2 (n=13), PanIN-3 (n=13), and PDAC (n=82) \cite{84}. In normal pancreas, LCN2 immunostaining was weak to moderate and confined to the duct cells within the cytoplasm and membrane, (mean score: 2.77 ± 0.42; Figure 2.1A). The staining pattern intensified in the small/medium sized ducts, and lumen of the larger ducts of high grade PanINs. The mean scores were 3.95 ± 0.55 for PanIN-1, 8.00 ± 0.88 for PanIN-2, and 8.27 ± 1.01 for PanIN-3. All PDAC tissue samples stained positive
for LCN2 expression (mean score: 5.93 ± 0.33). Significant differences in staining were observed between normal pancreas and PanIN-2 and -3 lesions, as well as normal compared to PDAC (Kruskal-Wallis one way ANOVA, p<0.001).

![Image of LCN2 expression in pancreatic neoplastic lesions and PDAC cell lines]

Figure 2.1. LCN2 expression in pancreatic neoplastic lesions and PDAC cell lines. (A) The LCN2 immunostaining pattern for normal (n=31), PanIN1 (n=22), PanIN-2 (n=13), PanIN-3 (n=13), and PDAC (n=82). Mean scores and the SEM for LCN2 immunostaining are noted below the sections. (B) LCN2 gene expression was examined in 21 different PDAC cell lines. Relative expression was normalized using loading controls and then normalized to the H6c7 ratio. (C) Representative immunoblots of LCN2 and GAPDH protein expression in PDAC cell lines.

2.4.2 LCN2 expression in PDAC cell lines.
After determining LCN2 staining in PanIN and PDAC samples, we next wanted to assess LCN2 mRNA expression in 21 PDAC cell lines. 80% of the cell lines displayed elevated expression compared to the normal H6c7 cell line (Figure 2.1B). However, MiaPaca2, PANC1, PK1, and PK8 PDAC cell lines showed minimal or no LCN2 expression compared to H6c7 cells (Figure 2.1C). By Western blot, protein expression levels were concordant with mRNA levels in the majority of the cell lines.
2.4.3 **Knockdown and overexpression of LCN2 in PDAC cell lines.**

We previously reported an increased LCN2 expression after KRAS\(^{G12V}\) expression in H6c7 cells [84]. This expression was maintained in the tumor cell line, H6c7KrT established from a tumor that developed subcutaneously after implantation of H6c7KRAS\(^{G12V}\) cells in SCID mice [84]. LCN2 mRNA expression was 10- and 2-fold higher in H6c7KRAS\(^{G12V}\) and H6c7KrT cells compared to the vector control H6c7-pBp cells [84]. Three separate shRNA sequences were used to downregulate LCN2 expression in H6c7KrT cells, resulting in reduction of LCN2 mRNA levels by 76%, 92%, and 65%, as compared to the non-silencing (NS) shRNA (Figure 2.2A). Protein lysates and conditioned media also showed concordant reduction in LCN2 protein expression (Figure 2.2B).

**Figure 2.2.** The knockdown and overexpression of LCN2 expression in PDAC cell lines. (A) LCN2 mRNA expression was suppressed using three different retroviral shRNA constructs (KD) in H6c7KrT cells. (B) Representative immunoblots of LCN2 protein expression in H6c7KrT cells, where GAPDH was used as a loading control. (C) LCN2 expression was downregulated in BxPC3 and HPAF-II cells. In PANC1 cells LCN2 was overexpressed by a lentiviral expression construct. (D) Representative immunoblots of LCN2 protein expression in BxPC3, HPAF-II, and PANC1 cells.
The LCN2KD2 shRNA sequence was selected for subsequent experiments as it yielded maximal knockdown of LCN2 expression. Stable transduction of LCN2KD2 and NS shRNA into the BxPC3 and HPAF-II cell lines decreased LCN2 mRNA expression by 78% and 87%, respectively (Figure 2.2C). We stably transduced the low LCN2 expressing PANC1 cells with a LCN2 expression construct or a control empty vector (EV). Q-PCR and immunoblot analyses confirmed the overexpression (Figure 2.2D). Secreted production of LCN2 was consistent with intracellular protein levels. Modifying LCN2 expression in PDAC cell lines did not alter cellular proliferation (Figure 2.3A-C).

Figure 2.3. **LCN2 does not alter cellular proliferation.** Cell growth curves for (A) BxPC3 NS and -LCN2KD2, (B) HPAF-II NS and -LCN2KD2, and (C) PANC1 EV and -LCN2 (n=3).

**2.4.4 LCN2 improves adhesion and invasion of PDAC cells.**

LCN2 has been reported to mediate attachment to the basement membrane [143]. To investigate if LCN2 promotes adhesion in PDAC, LCN2 was suppressed in the H6c7KrT, BxPC3, and HPAF-II cell lines. Knocking down LCN2 decreased attachment of cells on fibronectin and collagen coated plates compared to the NS control (one-way ANOVA for the KrT cell line and t-test for the BxPC3 and HPAF-II cell lines, p<0.05; Figure 2.4A, B). LCN2 overexpression increased adhesion in PANC1 cells compared to EV control (t-test, p<0.05).
Thus, LCN2 contributes to the adhesion of PDAC cells on fibronectin and collagen I substrata (t-test, p<0.01).

Figure 2.4. **LCN2 promotes adhesion and invasion in PDAC cells.** Adhesion assays were performed on the (A) H6c7 KrT, (B) BxPC3, HPAF-II, and PANC1 cell lines. Fold changes were calculated by comparing the KD to NS or LCN2 to EV (n=3). The fold changes in invasive ability were calculated by comparing the effects of the shRNA constructs against the NS control, or LCN2 overexpression compared to the EV control (n=3). Invasive ability was assessed in (C) H6c7KrT cells (n=3), (D) BxPC3, HPAF-II, and PANC1 cells (n=6) were seeded onto Matrigel or collagen IV coated membranes. MMP-9 gene expression and activity were assessed in BxPC3, HPAF-II, and PANC1 cells after modulating LCN2 expression by (E) Q-PCR. To assess MMP-9 activity gelatin zymography was performed on the conditioned media from (F) H6c7 KrT cells, (G) BxPC3, HPAF-II, and PANC1 cell lines. (H) Migration was assessed at 0, 24, and 48 hours after the scratch was made on confluent BxPC3 NS and –LCN2KD2, HPAF-II NS and –LCN2KD2, and PANC1 EV and –LCN2 cells. The percentage of cells migrating in the wound are as noted (n=3).

The binding of LCN2 to MMP-9 has been shown to prolong its enzymatic activity thereby enhancing invasion [124]. Invasion assays were performed to determine if LCN2 downregulation in H6c7KrT, BxPC3 and HPAF-II cells attenuated invasion through Matrigel and/or collagen IV coated membranes. LCN2 downregulation in H6c7KrT, BxPC3 and HPAF-II...
cells decreased invasion through Matrigel and/or collagen IV coated membranes. Each shRNA construct significantly diminished invasion by H6c7KrT cells through Matrigel by 71%, 77%, and 56%; and collagen IV by 72%, 80%, and 70% compared to the control, respectively (one-way ANOVA, p<0.01; Figure 2.4C). Knocking-down LCN2 in the BxPC3 and HPAF-II cell lines significantly reduced invasion through collagen IV by 60% and 70%, respectively (t-test, p<0.05). However, suppression of LCN2 affected only the ability of the HPAF-II cell line to invade through Matrigel (t-test, p<0.01; Figure 2.4D). Elevated LCN2 expression in PANC1 cells enhanced invasion through both substrata (t-test, p<0.05).

Gelatin zymography was performed to assess the interaction between LCN2 and MMP9. MMP-9 expression levels remained consistent after LCN2 modification (Figure 2.4E). Conditioned media was collected from the H6c7KrT, BxPC3, HPAF-II, and PANC1 cell lines to assess MMP-9 activity after LCN2 modification. LCN2 downregulation in H6c7KrT, BxPC3 and HPAF-II cell lines decreased MMP-9 activity by 30%, 66% and 88%, respectively. LCN2 expression in the PANC1 cell line caused a 5.4-fold increase in MMP-9 activity (Figure 2.4F, G). Altering LCN2 expression had no effect on migration (Figure 2.4H). Thus, LCN2 contributes to the invasiveness by promoting MMP-9 activity and attachment in PDAC cell lines.

2.4.5 LCN2 enhances gemcitabine resistance in PDAC cells in vitro.

Gemcitabine is used as a first-line chemotherapeutic agent in PDAC [12]. To determine if LCN2 promotes survival in PDAC, BxPC3, HPAF-II, and PANC1 cell lines were treated with gemcitabine or vehicle for 72 hours and assessed for cell viability by propidium iodide (PI) exclusion. Knocking-down LCN2 in the BxPC3 and HPAF-II cell lines significantly increased the number of PI-positive cells indicating cell death, while LCN2 overexpression in PANC1
cells conferred increased resistance to gemcitabine (t-test, p<0.05, Figure 2.5A). Immunoblot analysis of cleaved caspase-3 validated the flow cytometry results. Depleting LCN2 expression increased caspase-3 cleavage by two-fold in gemcitabine treated BxPC3 and HPAF-II cell lines, while LCN2 expression in PANC1 reversed this effect (Figure 2.5B). No significant differences in the expression of anti-apoptotic proteins Bcl-xL and Bcl-2, and pro-apoptotic proteins Bad, Bax, and Bim were observed after altering LCN2 expression. The half maximal inhibitory concentrations (IC50) of gemcitabine dependence on LCN2 were investigated. The IC50 concentrations of the control BxPC3, HPAF-II, and PANC1 were 10 µM, 50 µM, and 20 µM, respectively. Knocking down LCN2 in the BxPC3 and HPAF-II cell lines reduced the IC50 concentrations to 5 µM and 20 µM, respectively. Whereas, expression of LCN2 in PANC1 increased the IC50 to 50 µM of gemcitabine (n=5; Figure 2.5C-E).

Figure 2.5. *LCN2 promotes gemcitabine resistance in PDAC cells.* (A) PI exclusion assays for cell death (n=6) and (B) immunoblot analysis after 72 hours treatment by gemcitabine on the BxPC3, HPAF-II, and PANC1 cell lines (n=3). IC50 concentrations were assessed in (C) BxPC3 NS and –
2.4.6 *LCN2-associated global transcriptional changes.*

Several studies have identified LCN2 as an upregulated gene in cancer. However, no studies have yet examined the effect of LCN2 on gene expression. To examine how LCN2 affects gene expression in PDAC cell lines, transcriptional profiling was performed on the BxPC3 cell lines and xenografts. LCN2 expression upregulated the expression of 623 genes (Appendix Table 1) and downregulated the expression of 538 genes (Appendix Table 2). The putative LCN2 target genes were annotated to GO biological processes and were significantly enriched for processes involved in apoptosis (28/623; \( p=0.008 \)), cell cycle (32/623; \( p=0.02 \)), and adhesion (14/623, \( p=0.02 \)). The downregulated genes annotated to GO biological processes were significantly enriched for genes involved in apoptosis (36/538; \( p=0.004 \)).

The genes annotated to the apoptotic pathway were analysed according to gene function which revealed that 57% of the upregulated genes were involved in survival, and 67% of the downregulated genes were pro-apoptotic (Figure 2.6A). To validate this we performed Q-PCR in the BxPC3, HPAF-II, and PANC1 cell lines. We then verified the expression of a subset of these genes by QPCR. The pro-apoptotic gene AIFM1 was identified to have higher expression in the BxPC3 and HPAF-II cell lines after LCN2 was knocked-down (Figure 2.6C). Whereas, expressing LCN2 in PANC1 cells enhanced expression of anti-apoptotic genes BIRC2, FAIM, and MCL-1 compared to the control (\( p<0.05 \); Figure 2.6D-F).
Figure 2.6. **LCN2 promotes survival and adhesion.** (A) LCN2 enhances the expression of anti-apoptotic genes and downregulated the pro-apoptotic genes. (B) LCN2 enhances adhesion and ECM. Target genes whose expression was up/downregulated by at least 1.5-fold in the control cell line and xenograft samples compared to the LCN2 downregulated cell line and xenograft samples. Red triangles denote increased expression and green triangles denote decreased expression. The mRNA expression of (C) AIFM, (D) BIRC2, (E) FAIM, (F) MCL-1, (G) LAMAC2, (H) MMP7, (I) CDH11, and (J) ITGA2 were assessed in BxPC3, HPAF-II, and PANC1 cell lines. (* denotes significant differences between the test and control samples (p<0.05, student t-tests, n=3).

Additionally, the genes enriched for attachment were examined (Figure 2.6B). 44% of the genes promoted cell to cell attachment, whereas the remaining genes positively regulated cell
to ECM adhesion. Q-PCR validation demonstrated that expressing LCN2 in the PANC1 cell lines promoted expression of LAMAC2, MMP-7, CDH11, and ITGA2 (p<0.05; Figure 4G-J). Whereas depleting LCN2 expression in the BxPC3 and HPAF-II cell lines downregulated expression of MMP-7 and CDH11 (p<0.05; Figure 2.6H, I). We identified that LCN2 enhances expression of genes annotated to adhesion and survival in PDAC.

2.4.7 LCN2 promotes tumorigenicity in the xenograft model.

The expression of LCN2 has been demonstrated to enhance breast tumour formation and progression [125]. To determine how LCN2 affects tumorigenicity in PDAC, BxPC3, HPAF-II, and PANC1 cells were implanted subcutaneously into SCID mice. Knocking-down LCN2 in both BxPC3 and HPAF-II cells significantly reduced tumor growth when compared to the NS xenografts (p<0.01; Figure 2.7A, B). Consistently, PANC1 LCN2 cells had markedly increased tumor growth compared to the EV xenografts (p<0.01; Figure 2.7C). Gelatin zymography was employed to examine the effects of suppressing or overexpressing LCN2 on MMP-9 activity in PDAC xenografts. Diminishing LCN2 expression reduced MMP-9 activity by 35% and 79% in BxPC3 and HPAF-II xenografts, respectively (Figure 2.7D, E). There was little MMP-9 activity in the PANC1 xenografts, and LCN2 expression promoted MMP-9 activity in half of xenografts assessed (Figure 2.7F). Together, LCN2 expression influences tumourigenicity in vivo and promotes MMP-9 activity in PDAC cell lines that highly express LCN2.
2.4.8 LCN2 promotes gemcitabine insensitivity in resistant PDAC cells in vivo.

Since LCN2 has been demonstrated to promote survival in PDAC and several cancer cell line models in vitro, we wanted to determine if LCN2 would have an effect on gemcitabine sensitivity of PDAC in vivo [120,130]. Tumor bearing mice were treated with vehicle (PBS) or 100 mg/kg gemcitabine once every seven days. BxPC3 is inherently insensitive to gemcitabine, whereas PANC1 was sensitive in vivo [144]. Gemcitabine treated BxPC3 NS mice showed no change in tumor growth compared to the vehicle treated mice. Attenuating LCN2 expression in BxPC3 cells reduced tumor growth (p<0.0001) and increased sensitivity to gemcitabine (p=0.0003; Figure 2.8A; Table 2.2). In PANC1 cells, LCN2 expression enhanced tumor growth (p=0.00035; Figure 2.8B), but was not correlated with increased resistance to gemcitabine (p<0.00001). Gemcitabine treatment of mice bearing BxPC3 LCN2KD2 xenografts displayed a 5-fold increase in cleaved caspase-3 compared to treated control xenografts (Figure 2.8C;
p<0.0001). Whereas, expressing LCN2 in the PANC1 xenografts reduced caspase-3 cleavage after gemcitabine treatment by 30% (p=0.035). Assessment of LCN2 immunostaining revealed that the BxPC3 xenografts maintained the expression of the shRNA targeted against LCN2, and the PANC-1 xenografts retained expression of the LCN2 construct (Figure 2.8D). Furthermore, Ki67 immunostaining did not demonstrate any differences in proliferation between high and low LCN2 expressing xenografts. Thus, LCN2 promotes gemcitabine resistance in insensitive lines.
Figure 2.8. *LCN2 promotes resistance to gemcitabine and angiogenesis.* Effect of gemcitabine on the growth of tumors formed by (A) BxPC3-NS and −LCN2KD2 cell lines and (B) with PANC1-EV and −LCN2 cell lines (*denotes significance between vehicle treated mice, n=10 per group, p<0.0001, mixed model multiple regression, †denotes significance between vehicle and gemcitabine treated mice (n=10 per group, p=0.0003, mixed model multiple regression). (C) Protein lysates isolated from
BxPC3 and PANC1 xenografts were assayed for caspase-3 cleavage and LCN2 expression after gemcitabine treatment (n=10). β-actin served as a loading control (D) Representative histological images of BxPC3 NS and –LCN2KD2, and PANC1-EV and –LCN2 xenografts after H&E, and immunostaining for LCN2, Ki67, and murine CD31. (E) Vascular density in five hot spots at high magnification in the BxPC3 NS and –LCN2KD2, and PANC1-EV and –LCN2 xenografts. The mRNA expression of (F) HIF1A and (G) VEGF in the BxPC3 and PANC1 xenografts. Gene expression was compared between KD and NS, or LCN2 and EV. (*denotes significance between KD and NS, or LCN2 and EV, n=20, p<0.05, student t-test).

**Table 2.2. Differences in log growth rates after LCN2 modification and gemcitabine treatment**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Difference in growth rate (log values)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BxPC3 NS: Gemcitabine vs Vehicle</td>
<td>-0.02736</td>
<td>0.7816</td>
</tr>
<tr>
<td>BxPC3 KD: Gemcitabine vs Vehicle</td>
<td>0.5168</td>
<td>0.0003</td>
</tr>
<tr>
<td>Vehicle: BxPC3 KD vs NS</td>
<td>-0.5814</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gem.: BxPC3 KD vs NS</td>
<td>-0.03648</td>
<td>0.8147</td>
</tr>
<tr>
<td>PANC1 EV: Gemcitabine vs Vehicle</td>
<td>-0.026</td>
<td>0.00002</td>
</tr>
<tr>
<td>PANC1 LCN2: Gemcitabine vs Vehicle</td>
<td>-0.028</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Vehicle: PANC1 LCN2 vs EV</td>
<td>0.02</td>
<td>0.00035</td>
</tr>
<tr>
<td>Gem.: PANC1 LCN2 vs EV</td>
<td>0.019</td>
<td>0.0038</td>
</tr>
</tbody>
</table>

Tumour volume was log-transformed to stabilize variance and linear mixed effect model was used to determine differences in tumour growth. The differences in log growth rate and p-values are presented per paired comparison.

### 2.4.9 LCN2 promotes angiogenesis

HIF1A was identified as one of the significantly upregulated genes in the microarray analysis which prompted us to assess the vascularity and VEGF expression in the BxPC3 and PANC1 xenografts [134]. The quantification of CD31 positive blood vessels revealed that knocking-down LCN2 in BxPC3 cells decreased vascularity by 15%, and HIF1A and VEGF expression (Figure 2.8E-G). Whereas expressing LCN2 in PANC1 cells increased vascularity by
11% and elevated expression of these angiogenic genes in vivo (p<0.05). We conclude that LCN2 promotes tumor growth, invasion, angiogenesis, and maintains resistance to gemcitabine in insensitive lines.

2.5 Discussion

In the present study, the use of multiple modalities has provided a cohesive study into the function of LCN2 in PDAC and its pattern of expression during pancreatic carcinogenesis. We have shown that LCN2 expression is associated with the progression of PanIN lesions and PDAC. Through expression profiling studies, we have also demonstrated that LCN2 upregulates genes involved in survival, adhesion, and cell cycle, and downregulates pro-apoptotic genes. We have provided strong evidence that LCN2 promotes attachment, invasion, tumor growth, and gemcitabine resistance in multiple PDAC cell lines.

We were able to demonstrate by gelatin zymography that modifying LCN2 expression affects MMP-9 enzymatic activity. Depleting LCN2 abrogates invasion through basement membrane substrata, Matrigel, and collagen IV by PDAC cells. Since MMP-9 is a collagenase, depleting LCN2 in the BxPC3 and HPAF-II cell lines attenuated invasion through collagen IV. However, invasion through Matrigel was hindered in the BxPC3 cell line only. Matrigel is composed of other extracellular matrix proteins besides collagen such as laminins and proteoglycans, and represents a more complex substratum to invade through than collagen. In vivo, we observed that depleting LCN2 in BxPC3 and HPAF-II xenografts suppressed MMP-9 activity. Together, our findings demonstrate that LCN2 expression promotes MMP-9 activity and are consistent with other cancer cell types. LCN2 depletion in colon [143], gastric [128], and breast cancer models [125,129] diminishes MMP-9 activity thereby attenuating invasion. Furthermore, the presence of LCN2-MMP-9 complexes have been found in the urine of breast
cancer patients and in tissue homogenates of gastric cancer patients [122,127]. Clinically, detection of this complex can be used as a diagnostic predictor for breast cancer, has been associated with poor prognosis in gastric cancer, and was linked to depth of tumour invasion in esophageal cancer [122,127]. The presence of LCN2 protects MMP-9 from autodegradation which sustains its activity [124].

LCN2 stimulates tumour growth in vivo. In this study, enhancing LCN2 expression in PANC1 cells markedly increased tumor growth, whereas suppressing its expression in BxPC3 and HPAF-II cells reduced tumor growth rate. Similar findings have been reported in several breast cancer studies that have demonstrated the critical role of LCN2 expression in the aggressive growth of tumours [122,125,129,145] and metastatic dissemination [125,129,145]. Tumour growth has been associated with enhanced VEGF expression in endometrial cancer [146]. Moreover, we identified that LCN2 promoted HIF1A and VEGF expression in several PDAC cell lines which contributed to an increase in vascularity in vivo. Modifying LCN2 expression had no effect on cellular proliferation as evidenced by Ki67 immunostaining and in culture. Nor were there any differences in caspase-3 cleavage in vehicle treated animals. These data suggest that LCN2 does not affect proliferation or survival in vehicle treated animals. Altogether, our data supports the association of LCN2 expression and aggressive tumour growth by stimulating angiogenesis through the upregulation of HIF1A and VEGF.

LCN2 has been shown to suppress apoptosis in thyroid, lung, and breast cancers, thus we wanted to ascertain if LCN2 promoted survival in PDAC [120,130]. Low LCN2 expressing cell lines had increased cleaved caspase-3 and PI-inclusion after gemcitabine treatment in vitro. Furthermore, depleting LCN2 in the gemcitabine resistant BxPC3 cell line restored sensitivity and increased caspase-3 cleavage in vivo. Several recent studies have implicated LCN2 as an anti-apoptotic protein by blocking the activation of caspase-9 through Bim [120,130]. Though
changes in Bim expression were not noted, we did find differences in apoptosis related gene expression in the microarray. Analysis of the apoptosis enriched fraction revealed that LCN2 expression upregulated the expression of survival genes such as BIRC2, FAIM, and MCL-1. BIRC2 and MCL-1 have been previously identified to be overexpressed in PDAC and are associated with PDAC progression [147,148]. Whereas, knocking down LCN2 resulted in increased expression of pro-apoptotic AIFM1, which is involved in caspase-dependent and -independent apoptosis [149]. Together, these data demonstrate that LCN2 has a role in cell survival and resistance in drug-induced apoptosis. In summary, we provide evidence utilising several cell lines that LCN2 promotes tumorigenicity in PDAC by enhancing invasion, tumour growth, angiogenesis, and gemcitabine resistance.

Contradicting our findings and other studies, Tong et al. suggested that LCN2 acts as a tumor suppressor gene in PDAC [134]. They reported that using a single shRNA to downregulate LCN2 expression in the high LCN2 expressing BxPC3 cells led to increased invasion and attachment. While forced LCN2 expression in the low LCN2 expressing MiaPaca2 cell line decreased tumour size, metastatic spread, VEGF expression, and microvascular density [134]. Tong and colleagues observed that LCN2 did not confer any protection against gemcitabine after 48 hours of in vitro treatment, and they did not study gemcitabine sensitivity in vivo [134]. We also observed no differences within 48 hours of treatment in vitro, but after 72 hours of gemcitabine treatment we found that LCN2 expressing cell lines were more chemoresistant which is consistent with evidence in other experimental models where LCN2 acts as an anti-apoptotic protein [120,130]. Contrary to our findings, Tong et al. reported that LCN2 decreases VEGF expression, HUVEC tubule formation, and microvascular density in MiaPaCa2 xenografts [134]. In our study, we observed in two PDAC xenograft models a positive association between LCN2 expression, and expression of VEGF and HIF1A which promoted tumour vascularity.
Furthermore, we found that high expression of LCN2 corresponded with increased tumourigenicity in three PDAC cell line xenograft models. Whereas Tong and colleagues found contrasting observations in the MiaPaCa2 cell line only [134]. In contrast to our study, Tong et al reported that knocking down LCN2 in the BxPC3 cell line increased invasiveness and attachment. Whereas, we demonstrated that suppressing LCN2 expression in three PDAC cell lines decreases attachment and expression of genes that promote attachment, and invasion by attenuating MMP-9 activity. Despite the many studies that have highlighted the interaction between LCN2 and MMP-9 which promotes invasion [122-126] Tong and colleagues did not explore this key interaction in their study [134]. It is difficult to reconcile the disagreement between our findings and the Tong et al. study, thus further studies by independent laboratories may be warranted [133,134,150].

PDAC is one of the most fatal cancers with a very poor prognosis [136] and we have provided evidence on the importance of LCN2 in contributing to aggressive and drug resistant phenotypes. Currently CA19-9 is the most commonly used serum biomarker to diagnose PDAC [12]. Recently, elevated serum LCN2 has been identified in acute pancreatitis patients [139]. Increase in LCN2 expression has been reported in renal injury, inflammation, and other cancer types [125]. The elevation of LCN2 in these numerous conditions indicates that regulation of its expression is varied which reflects its multi-faceted function. The expression of LCN2 was found to be ubiquitously expressed in PDAC patient samples and its elevated expression was associated with high-grade lesions. LCN2 has been described to be a potential biomarker for cancer progression as it has been found in the urine of breast cancer patients and in the serum of PDAC patients [122,151]. Thus, LCN2 may represent a new biomarker for early diagnosis, prognostication and therapeutic targeting in PDAC.
Chapter 3

**Expression of KRAS and incomplete knock down of Smad4 is insufficient to transform human pancreatic duct epithelial cells**

Lisa Leung, Nikolina Radulovich, and Ming-Sound Tsao.

Author contributions (percentage of contribution):

LL: conceived the study, conducted the *in vitro* and *in vivo* experiments, performed the statistical analysis, and wrote the manuscript (95%)

NR: assisted in the cloning of the KRAS expression construct and cell line generation (5%)
3.1 **Abstract**

Pancreatic cancer is the fourth most common cause of cancer death in North America. Over 90% of tumours arise from the pancreatic duct and are classified as ductal carcinoma. PDAC evolves through a multistage neoplastic transformation process characterised by the advancement of histologically well-defined precursor lesions and cumulative genetic aberrations. In PDAC, KRAS oncogene activation has been observed in 90% of cases, while the inactivation of tumour suppressor gene Smad4 occurs in 55% of cases. Introduction of multiple genetic aberrations into normal pancreatic duct cells may provide a dynamic model to study pancreatic duct cell carcinogenesis. Smad4 was stably inactivated by shRNA to study its effect and interaction with KRAS$^{G12V}$ in the H6c7 cell line. Knocking down Smad4 disrupted TGF-β responsiveness and repressed Smad4 responsive genes expression. KRAS$^{G12V}$ expression or Smad4 depletion enhanced invasion through Matrigel. Depleting Smad4 in the H6c7-KRAS cell line led to stochastic tumour formation in one out of five mice tested. This study demonstrates that significant, but incomplete Smad4 inactivation is insufficient to cooperate with the KRAS oncogene in the malignant transformation of HPDE cells.
3.2 **Introduction**

PDAC evolves through a multistage neoplastic transformation process characterised by histologically well-defined precursor lesions termed pancreatic intraepithelial neoplasias (PanINs) [13]. The molecular progression model of PDAC has linked the progression of PanINs with cumulative genetic aberrations. [13]. In the model, the KRAS oncogene is activated in 90% of cases, and the inactivation of the p16, p53, and Smad4 tumour-suppressor genes are found in 95%, 75%, and 55% of PDAC cases, respectively [33]. Transforming pancreatic duct cells with multiple genetic aberrations can provide a model for understanding how each of these mutations affects pancreatic carcinogenesis.

The early and near ubiquitous activation of KRAS implicates a role for this gene in the pathogenesis and biology of this disease [13]. Oncogenic KRAS constitutively activates downstream pathways involved in cell survival, motility, and proliferation [16]. Targeted KRAS$^{G12D}$ expression in the mouse pancreas induces PanIN lesions that can gradually progress to PDAC. These mouse models provide evidence that KRAS activation is a tumour-initiating event, however further events are required for the acceleration of PDAC progression [49].

Smad4 plays a crucial role as a common binding partner to activated receptor Smads, Smad2 and -3, in the canonical TGF-β signalling pathway. The TGF-β signalling pathway is frequently disrupted in pancreatic cancer. Smad4 has been demonstrated to be dispensable for pancreatic development and does not initiate pancreatic neoplasms [54]. However, targeted Smad4 loss and KRAS$^{G12D}$ expression in the mouse pancreas leads to the rapid development of cystic tumours and mPanIN lesions [54,63,64]. The human pancreatic duct epithelial cell line, H6c7, can be utilized for dynamic modeling of pancreatic carcinogenesis. We report here that the effect of a partial but significant knockdown of Smad4 in the H6c7 cell line in the presence and
absence of KRAS$^{G12V}$ promotes invasion, but is insufficient for transformation of the H6c7 cell line.

3.3 Materials and Methods

**Cell culture.** Human pancreatic duct epithelial cell line, H6c7, H6c7 NS, H6c7 S4KD1-4, H6c7 KRAS, and H6c7 KRAS S4KD2 were grown in keratinocyte growth medium (KGM) media supplemented with bovine pituitary extract, hEGF, insulin, hydrocortisone, epinephrine, and transferrin (Lonza, Basel, Switzerland). Anchorage independent growth was assessed by suspending 1000 cells in KGM media supplemented with 0.3% Bacto-agar (Difco, Detroit, USA). The cell agar layer was overlaid a 1.2% Bacto-agar layer. Proliferation was assessed for 8 weeks.

**Smad4 small interfering RNA gene silencing.** Smad4 expression was stably downregulated by shRNA retroviral transduction method. The shRNA sequences were ligated into the pSUPER GFP retrovirus vector after linearization with BglII and HindIII (New England Biolabs, Pickering, Canada). The shRNA oligonucleotides used were: S4KD1: ggacaatagttattacgaa; S4KD2: gcagtgactttgtatagagaa; S4KD3: actgctaaattctatgttaaa; S4KD4: ggtggagagagtgaaacattt; and non-silencing control siRNA sequence: ttctcgaacgtgcact (Qiagen, Dusseldorf, Germany). The shRNA retroviral expression vectors were generated using Phoenix-amphotropic packaging cell line (ATCC).
**Quantitative real-time RT-PCR.** Total RNA isolation and PCR was performed as described before [140]. Primer sequences are listed in the table below.

Table 3.1. **QPCR primer sequences**

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<th>Reverse</th>
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<td>gttctgtctaaagctccttg</td>
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<td>ACTB</td>
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<td>gggagagacagtggccatt</td>
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</tbody>
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**TGF-β growth assay.** Twenty thousand cells were seeded in triplicate in a 6-well dish. To determine the anti-proliferative effect of TGF-β (R&D Systems), cells were treated with 10 ng/ml TGF-β on the second day after seeding. Cells were counted at days 2, 4, 6, and 8 on a Beckman Coulter Counter (Beckman).

**Tumorigenic assay.** All animal studies were carried out using protocols that have been approved by the Animal Care Committee. Tumorigenicity in severe combined immunodeficient (SCID), non-obese diabetic SCID (NOD SCID), and NOD SCID gamma (NSG) mice were assayed by subcutaneous injection. Two million cells were suspended in 70 µl serum-free medium supplemented with 10% matrigel.

**Western blot.** Whole cell extracts were applied to SDS polyacrylamide gels and assayed for KRAS (Oncogene), phospho-Akt (Thr308) (Cell Signaling), phospho-MAPK (Cell Signaling), Smad4 (Santa Cruz Biotechnology), and GAPDH (Abcam). KRAS activity was assessed by
using the RAS activation kit (Upstate). Visualization was accomplished by using horseradish peroxidase-linked anti-rabbit and anti-mouse secondary antibodies (Cell Signaling) and ECL-Plus blotting substrate detection kit (Amersham) on a Typhoon phospho- and fluorescent imaging system (Amersham). Resulting blots were analyzed with ImageJ software (NIH, Bethesda, MA).

**Invasion Assay.** Fifty thousand cells were seeded onto a 24-well Transwell cell culture chambers (BD Biosciences) fitted with multiporous (8 µm pore size) polycarbonate membranes. The upper chambers of the membrane were coated with 13 µg of matrigel or 7.5 µg of collagen IV and the lower chamber was filled with 800 µl of media supplemented with 5 µg/ml of fibronectin. After 48 hours, cells were fixed with 0.5% glutaraldehyde and stained with 0.5% crystal violet reagent for 48 hours. Cells in the upper chamber were removed and the membrane was mounted onto glass slides for scanning. Migratory cells were counted with Aperio ImageScope software. Each cell line was assayed in heptaplicate.

**Immunohistochemistry.** Immunohistochemistry was performed based on previously described methods [142].

3.4 **Results**

3.4.1 **Smad4 knockdown.**

To assess the consequences of Smad4 deficiency in normal pancreatic duct epithelial cells, H6c7 cells were stably transduced with four different retroviral Smad4KD (S4KD)
constructs and a non-specific (NS) shRNA construct. QPCR revealed that the four constructs knocked down Smad4 expression by 60%, 80%, 51%, and 37%, respectively (Figure 3.1A). Western blotting demonstrated concordance between the mRNA and protein expression (Figure 3.1B). The second shRNA sequence was selected for subsequent experiments as it yielded maximal knockdown of Smad4 expression. Depleting Smad4 in the H6c7 cell lines did not affect the expression of other canonical TGF-β signalling members (Figure 3.1C).

Figure 3.1. Stable Smad4 knockdown does not affect expression of other TGF-β signalling components. (A) Smad4 mRNA was suppressed using four different shRNA constructs (KD1-4) and a nonsense (NS) in the H6c7 cell line. (B) Representative Western blots of Smad4 protein expression in Hc67 cells, where GAPDH is used as a loading control. (C) The mRNA expression levels of TBRI, TBRII, Smad2, and Smad3.
3.4.2 **KRAS activation.**

Knocking down Smad4 had no effect on KRAS gene expression. KRAS$^{G12V}$ was stably expressed by 7-fold in the H6c7 (H6c7-KRAS) compared to the empty vector control (Figure 3.2A). Smad4 expression was similarly knocked down by 80% in the H6c7-KRAS cell line (Figure 3.2B). The increased KRAS mRNA expression was associated with an increase in KRAS protein expression (Figure 3.2C). Downstream AKT and MAPK activation were not triggered despite the presence of KRAS-GTP (Figure 3.2D). Introduction of KRAS$^{G12V}$ had no effect on the expression of TGF-β signalling members (Figure 3.2E).

![Figure 3.2](image-url)

**Figure 3.2. Stable KRAS$^{G12V}$ expression and Smad4 knockdown.** (A) KRAS mRNA expression in H6c7 after introduction of NS and S4KD2. (B) Smad4 mRNA expression was suppressed after using S4KD2 shRNA construct. (C) Western blots of Smad4 and KRAS expression. (D) Western blots of activated RAS, phospho- and total AKT, and phospho- and total MAPK. GAPDH was used as a loading control. (E) The mRNA expression levels of TBRI, TBRII, Smad2, and Smad3.
3.4.3 \textit{KRAS}^{G12V} \textit{expression and/or Smad4 knockdown does not affect growth properties of H6c7 cells.}

Smad4 downregulation and/or \textit{KRAS}^{G12V} expression did not affect proliferation of H6c7 cells (Figure 3.3A, B). Anchorage independent growth was not observed after \textit{KRAS}^{G12V} introduction and/or knocking down Smad4.

**Figure 3.3.** Knocking down Smad4 or \textit{KRAS}^{G12V} expression does not alter cell growth or upstream activation of Smad2/3. Growth curves of (A) H6c7-NS and H6c7-S4KD2 (B) H6c7, H6c7-KRAS-S4KDNS, and H6c7-KRAS-S4KD2. Cells were treated with TGF-β on Day 2. The mRNA expression levels of (C) PAI-1 and (D) SMAD7 after 48 hours of TGF-β stimulation in the H6c7-NS, H6c7-S4KD2, H6c7-KRAS, H6c7-KRAS-NS, and H6c7-KRAS-S4KD2 cell lines. (E)
Western blots of phospho- and total Smad2, phospho- and total Smad3, and Smad4. GAPDH was used as a loading control.

3.4.4 Smad4 depletion promotes TGF-β insensitivity.

Smad4 plays an integral role in the canonical TGF-β signalling pathway. Stimulating the NS cell lines with 10ng/ml TGF-β suppressed cellular proliferation by 70% at the end of treatment (one way ANOVA, p<0.001). H6c7 cells became refractory to TGF-β treatment after Smad4 depletion (one way ANOVA, p<0.01; Figure 3.3A, B). KRAS<sub>G12V</sub> expression did not alter TGF-β response. Stimulating control and KRAS expressing cells with 10 ng/ml TGF-β for 48 hours increased Smad7 and PAI-1 mRNA expression, however this effect was blunted after knocking down Smad4 (one way ANOVA, p<0.05; Figure 3.3C-D). Residual Smad4 expression contributed to the diminished response of these TGF-β responsive genes.

3.4.5 SMAD4 depletion or KRAS<sub>G12V</sub> expression enhances invasion.

KRAS<sub>G12V</sub> expression or knocking down Smad4 increases H6c7 cell line invasiveness through Matrigel coated Boyden chambers (Figure 3.4A). Depleting Smad4 in the H6c7-KRAS cell line did not confer enhanced invasive ability. TGF-β is known to stimulate invasion and induce EMT in epithelial cells. Stimulating H6c7-NS cells with TGF-β increased the number of invasive cells by 6-fold, however this effect was not seen after suppressing Smad4 or KRAS<sub>G12V</sub> expression (Figure 3.4B).
Figure 3.4 KRAS$^{G12V}$ expression or knocking down Smad4 enhances invasion in H6c7 cells. (A) Invasion assays on membranes coated with Matrigel (n=6) incubated without and with (B) TGF-β.

The mRNA expression of (C) E-Cadherin, (D) Vimentin, (E) MMP2, (F) MMP9, and (G) Snail expression with and without TGF-β treatment.

The consequences of knocking down Smad4 or KRAS$^{G12V}$ expression on genes involved in invasion and epithelial phenotype were assessed. Oncogenic KRAS reduced basal expression of E-cadherin (Figure 3.4C). Silencing Smad4 increased endogenous expression of Vimentin (Figure 3.4D). TGF-β stimulation increased MMP-2 expression in all cell lines (Figure 3.4E). Enhanced MMP-9 expression was observed in the H6c7-NS and H6c7-S4KD2 cell lines, but was
absent in KRAS\textsuperscript{G12V} expressing cells (Figure 4F). Snai1 expression was highly induced after TGF-\(\beta\) stimulation in the H6c7-NS cell line, but was absent after silencing Smad4 and/or with expression of KRAS\textsuperscript{G12V} (Fig. 4G).

3.4.6 **Tumourigenic ability of the H6c7-KRAS-S4KD2 cell line.**

H6c7-pBp, H6c7-NS, H6c7-KRAS, H6c7-KRAS-NS, and H6c7-KRAS-S4KD2 cell lines were subcutaneously implanted into the flank of SCID and NOD SCID mice. No palpable masses were detected in SCID or NOD SCID mice after implantation. The lack of xenograft formation by the H6c7-KRAS or H6c7-KRAS-S4KD2 prompted the use of a more immunodeficient strain, NOD SCID gamma (NSG). The H6c7-NS, H6c7-S4KD2, H6c7-KRAS-NS, and H6c7-KRAS-S4KD2 cell lines were subcutaneously implanted into NSG mice (Table 3.2.). However, only the H6c7-KRAS-S4KD2 cell line was able to form detectable masses in four of the five NSG mice. One of these masses led to tumour formation that was first identified 65 days after injection and reached endpoint 88 days after initial detection (Figure 3.5A). Histological examination of the tumour revealed poorly differentiated carcinoma (Figure 3.5B). The other three mice carried small nodules which did not change in size after initial detection. The xenograft maintained expression of the shRNA and KRAS as demonstrated by immunoblotting (Figure 3.5C).
Figure 3.5. The expression of KRAS\textsuperscript{G12V} and knocking down Smad4 can incompletely transform H6c7 cells. (A) A tumour growth curve of the H6c7-NS, H6c7-S4KD2, H6c7-KRAS-NS, and H6c7-KRAS-S4KD2. (B) Histological image from the H6c7-KRAS-S4KD2 #5 (KS5) xenograft. (C) Western blots of Smad4 and KRAS in the H6c7 cell line and KS5 xenograft. β-actin was used as a loading control.

Table 3.2. Cell line phenotypes after Smad4 knockdown and/or KRAS\textsuperscript{G12V} expression

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumourigenicity</th>
<th>Invasion</th>
<th>Proliferation</th>
<th>TGF-β sensitive</th>
</tr>
</thead>
<tbody>
<tr>
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<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
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<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>0/5</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Kras Smad4KD2</td>
<td>1/5</td>
<td>++</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

Tumourigenicity was assessed in NOD-SCID gamma mice and the number of mice assessed are as listed. -, indicates no change or no effect; and +, indicates an effect or response.
3.5 Discussion

By examining single alterations and the combination of Smad4 depletion and KRAS$^{G12V}$ expression has permitted observation of the phenotypic changes to the H6c7 cell line. Smad4 depletion in combination with KRAS$^{G12V}$ expression incompletely transformed the normal human pancreatic duct epithelial cell line, H6c7. Knocking down Smad4 reduced TGF-β sensitivity. Diminishing Smad4 or introducing oncogenic KRAS expression did not alter proliferation rates in H6c7 cells, but instead increased invasion through Matrigel (Table 3.2).

We previously reported in our H6c7-KRAS ecotropic system that KRAS$^{G12V}$ increased AKT and MAPK activation [84]. Despite increased KRAS-GTP levels in the H6c7-KRAS cell lines, we were unable to observe similar findings in our amphotropic system. Similarly, PDAC cell lines, AsPC-1, Capan-1, HPAF-II, and Hs766T, which express mutated KRAS have low MAPK activation [152]. Analogous findings have been found in normal bronchial epithelial cells after KRAS$^{G12V}$ expression [153]. Elevated expression of MAPK phosphatase-2 and -3 in response to mutant KRAS expression has been implicated for reduced MAPK activity [87,153,154]. Hence, the lack of downstream stimulation of the MAPK and AKT pathways is not uncommon in PDAC.

Differences in tumourigenicity were also noted between the amphotropic and the ecotropic system [84]. Initial work examining KRAS mediated transformation of the H6c7 cell line employed the ecotropic packaging system. The KRAS oncogene is a biohazard and when these initial experiments were conducted the laboratory was not a biosafety level 2-plus (BL2+). Thus, we were not allowed to deliver an oncogene capable of infecting human cells. Ecotropic retroviruses can infect dividing murine or rat cells. The H6c7 cell line was constructed to
express an ecotropic retroviral receptor and was consequently employed to develop the H6c7-eco-pBp and H6c7-eco-KRAS\textsuperscript{G12V} cell lines. Following the availability of the BL2+ in our laboratory, we used amphototropic retroviruses which can infect dividing human cells [155]. While, H6c7-eco-Kras cells were able to form tumours after subcutaneous or orthotopic implantation in 50% of SCID mice, we were not able to replicate a similar result in the H6c7-KRAS cell line [84]. H6c7 cells transduced with ecotropic retrovirus receptor developed a tetraploid population. The inactivation of p53 and Rb after immortalisation likely permitted the occurrence of tetraploidy [156]. This tetraploid population likely created a more permissive environment for transformation after KRAS\textsuperscript{G12V} introduction since tetraploidy allows cells to sustain and accumulate mutations necessary for malignant transformation [157]. Despite differences in the viral delivery of KRAS\textsuperscript{G12V}, histological analysis of the H6c7-KRAS-S4KD2 xenograft revealed poorly differentiated carcinoma similar to what was observed in the H6c7-eco-KRAS xenografts [84]. Residual Smad4 expression may have conveyed its tumour suppressive activity which prevented complete transformation. Furthermore, usage of a more immunodeficient mouse strain may have facilitated better engraftment of the H6c7-KRAS-S4KD2 cell line. Engraftment efficiency is enhanced in the NSG strain since it lacks NK cell activity and is deficient in cytokine signaling as compared to the SCID and NOD-SCID strains which only lack T and B cells [158]. Thus, oncogenic KRAS and low Smad4 expression can partially transform the H6c7 cell line and stochastic tumour formation was observed in the NSG strain.

TGF-β mediated invasion has been reported to be dependent on Smad4 expression [90,159], our findings and other studies have shown that this may not apply to pancreatic cell lines. The enhanced invasiveness that was observed after knocking down Smad4 in the H6c7 cell line has also been seen in the Nestin positive human pancreatic epithelial cell [103].
Restoring Smad4 expression in the pancreatic cancer cell lines, BxPC3 and MiaPaCa-2, decreases invasion [105,160]. These data are consistent with the observations in pancreatic cancer patients where Smad4 loss was highly correlated with widespread metastatic disease [161]. Smad4 has been shown to be essential for the regulation of key proteins involved in migration such as downregulation of E-Cadherin, and upregulation of Snai1, Snai2, and Twist in PANC1 cells [162]. Depleting H6c7 cells of Smad4 did not alter E-Cadherin, MMP-2, or MMP-9 gene expression, but did increase Vimentin expression. KRAS\textsuperscript{G12V} expression reduced E-Cadherin expression. Stimulation of the control cell lines with TGF-β induced expression of Vimentin, MMP-2, and MMP-9 consistent with other studies that have demonstrated its role in EMT [37]. The significant decrease in Smad4 expression likely compromised the Smad-dependent TGF-β tumour suppressive signaling pathway. How Smad4-independent pathways promoted invasion in the H6c7 and PDAC cell lines needs to be further elucidated.

In summary, we have shown that Smad4 knockdown and KRAS\textsuperscript{G12V} expression contributes to increased invasion in the H6c7 cell line. However, residual Smad4 expression may have contributed to the incomplete transformation of the H6c7 cell line. In addition, use of the amphotropic system to stably express oncogenic KRAS may not have introduced genomic instability. Thus, further genetic alterations or complete loss of Smad4 expression may be required for tumorigenic transformation of the H6c7 cell line.
Chapter 4

Expression of KRAS$^{G12V}$ and loss of Smad4 contribute to the transformation of pancreatic duct carcinogenesis

Lisa Leung, Chang-Qi Zhu, Chang J Park, Christine To, Nikolina Radulovich, Emin Ibrahimov, and Ming-Sound Tsao

Author contributions (percentage of contribution):

LL: conceived the study, conducted the in vitro and in vivo experiments, performed the statistical analysis, and wrote the manuscript (80%)

CZ, CJP, and CT: assisted in the analysis of the aCGH and expression microarray (15%)

NR: assisted in the cloning of the KRAS expression construct and cell line generation (4%)

EI: performed the orthotopic implantation (1%)
4.1 Abstract

Pancreatic cancer is the fourth most common cause of cancer death in North America [136]. Over 90% of tumours putatively arise from the pancreatic ducts, and are thus called pancreatic ductal adenocarcinoma (PDAC). In PDAC, activating KRAS mutations and Smad4 tumour suppressor inactivation occur in 90% and 55% of cases, respectively [33]. The precise roles of oncogenic KRAS and Smad4 loss in the transformation of normal human duct cells have not been fully explored. Here we report that in a near normal human pancreatic duct epithelial (H6c7) cell line oncogenic KRAS expression and Smad4 inactivation is obligatory for tumourigenic transformation. Smad4 loss and KRAS$^{G12V}$ expression in an H6c7 derivative line gives rise to tumor growth and metastases in NOD-SCID mice. Restoring Smad4 expression increases tumour latency and decreases metastatic spread. Our data demonstrates that re-establishment of TGF-β signaling can reverse malignant behavior of cells and that Smad4 loss drives pancreatic cancer progression. Usage of this model will be important for future studies into initiation of pancreatic carcinogenesis.
4.2 Introduction

Pancreatic cancer is the fourth leading cause of cancer death in North America with an overall five year survival rate of less than 5% [136]. Over 90% of pancreatic tumours arise from the pancreatic ducts, and are thus known as pancreatic ductal adenocarcinomas (PDACs). PDAC evolves through a multistage neoplastic transformation process characterised by histologically well-defined precursor lesions termed pancreatic intraepithelial neoplasias (PanINs) [13]. The advancement of these lesions has been observed to be coupled to the cumulative aberrations in several cancer-associated genes leading to the development of the molecular progression model of PDAC [13]. In the molecular progression model, activating KRAS mutations have been observed in 90% of cases. Following KRAS activation are the loss in function of p16, p53, and Smad4 tumour-suppressor genes which occur in 95%, 75%, and 55% of PDACs, respectively [33]. By transforming pancreatic duct cells with multiple genetic aberrations, we can provide a model for pancreatic carcinogenesis to further understand how each of these molecular aberrations contributes to tumourigenesis.

The frequency of KRAS mutations in PDAC implicates a vital role for this gene in the pathogenesis and biology of this disease [13]. Point mutations that render KRAS in a constitutively active state stimulate downstream pathways involved in cell survival, motility, and proliferation [16]. Pancreas specific KRAS$_{G12D}$ knock-in induces mPanIN lesions formation that may progress to PDAC. This suggests that KRAS activation is a tumour-initiating event and that further events are required to accelerate PDAC progression [163].

Smad4 plays a crucial role as a common binding partner to activated receptor Smads, Smad2 and -3 in the canonical TGF-β signaling pathway. Briefly, binding the TGF-β ligand to the receptor complex leads to the phosphorylation of receptor activated Smads. Phosphorylated
Smads can then oligomerise with Smad4 to form part of a transcriptional complex which regulates processes such as cell cycle progression and extracellular matrix protein expression [164]. The TGF-β signalling pathway is frequently disrupted in pancreatic cancer and highly advanced carcinoma is associated with Smad4 loss [13,165]. Targeted Smad4 inactivation in the developing mouse pancreas does not initiate pancreatic cancer [54]. However, concomitant Smad4 loss and KRAS$^{G12D}$ expression in the mouse pancreas rapidly develops cystic tumours and mPanIN lesions [54,63,64].

We have previously reported that KRAS$^{G12V}$ expression in the human pancreatic duct epithelial (HPDE) cell line leads to tumourigenic transformation [84]. In chapter 3, shRNA targeted against Smad4 were utilised in the H6c7 cell line to examine the contribution of Smad4 loss. Significant downregulation of Smad4 expression increased invasiveness in H6c7 cells, but co-expression of KRAS$^{G12V}$ only stochastically led to the transformation of the H6c7 cell line. We have isolated another cell line from the H6c7 cell line called TGF-β resistant (TβR) which lacks Smad4 expression. In this study employment of a Smad4 null H6c7 cell line represented an ideal system to characterise Smad4 function in pancreatic duct cell carcinogenesis. Smad4 loss coupled with oncogenic KRAS results in the transformation of the H6c7 cell line with complete penetrance. Restoring Smad4 expression in the KRAS$^{G12V}$ expressing TβR cell line increased latency in tumour formation and reduced the number of metastases in vivo.

### 4.3 Materials and Methods

**Cell culture and in vitro assays.** H6c7, TβR, TβR-pBp, TβR-KRAS, TβR-KRAS-EV, TβR-KRAS-Smad4, TβR-pBp-EV, and TβR-pBp-Smad4 were grown in KGM media (Lonza, Basel,
Switzerland) as previously described [84]. To assess TGF-β sensitivity, cells were treated with TGF-β (R&D Systems) as previously described [84]. Invasion assays were performed as previously described [140]. Cell cycle analysis was performed by seeding 200 000 cells in 6-well dishes incubated with 1 μM aphidocolin for 18 hours. Cells were then stimulated for 24 hours with media. After stimulation cells were harvested, fixed in cold 70% ethanol overnight, stained with propidium iodide (Sigma), and analyzed on a BD FACScan (BD).

**Smad4 and KRAS<sup>G12V</sup> expression.** Stable Smad4 gene expression was achieved using the Gateway recombination cloning system [141]. Briefly, the Smad4 expression construct was purchased from Open Biosystems (Lafayette, CO). The full length cDNA was subcloned into the pDONR construct and recombined with an EGFP destination vector, which produced the plko.Smad4-EGFP vector. The vector was stably transduced into TβR cells by lentiviruses. Stable KRAS<sup>G12V</sup> expression was achieved by retroviral vector pBabepuro. The vector was stably transduced into the TβR cells by the Phoenix amphotropic packaging system as previously described [155].

**Quantitative real-time RT-PCR.** Total RNA was isolated from cells and PCR was performed as described before [140]. For Smad4 gene copy number, genomic DNA (gDNA) was isolated from cells using the DNAeasy kit (Qiagen, Toronto, Ontario). The individual primers are listed below.
Table 4.1. Primer Sequences

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**Amplification and sequencing of Smad4.** Sequencing primers and PCR amplification were performed as described previously [166]. PCR amplification of exons 1-11 was performed on gDNA. PCR products were enzymatically cleaned using ExoSAP-IT (Affymetrix, Santa Clara, CA). Sequenced PCR products were visualized and analysed using Chromas software (Technelysium, Eden Prairie, MN).

**Methylation specific PCR.** Total gDNA was isolated from cells and converted to bisulfite treated gDNA using the EpiTect bisulfite kit (Qiagen, Toronto, Ontario). Bisulfite treated gDNA was amplified using primers designed using the MethPrimer program [167]. Primer sets are as follows methylated (F: ttatagggctgtctttatagctg; R: catcactttttttttttttttctgc); Unmethylated (F: tatactttgtgtttatatggtg; R: catcaacttttttttttttttttc). PCR reactions were carried out using AmpliTaq (Applied Biosystems, Carlsbad, California) in 25 μL reaction volumes using the following conditions: 5 minutes initial denaturation 95°C, and 35 cycles of 95°C × 30s, 60°C × 30s, 72°C × 30s.
**Tumorigenic assay.** All studies were conducted under the auspices of the OCI Animal Care Committee. Tumor growth and implantation was assessed as described before [140]. Briefly, 2 million cells were suspended in total volume of 50 µl of KBM medium supplemented with 10% or with 20% Matrigel for subcutaneous or orthotopic injection into NOD SCID mice, respectively. Mice were euthanized according to animal protocol guidelines once subcutaneous tumours reached a diameter of 1.5 cm, or if mice presented with deteriorating clinical condition (abdomen distension, weight loss exceeding 20% of normal body weight, and hunched appearance).

**Western blot.** Western blotting was performed as previously described [140]. Briefly, whole cell extracts were applied to SDS polyacrylamide gels and assayed for KRAS, PAI-1, Smad4 (Santa Cruz Biotechnology); phospho- and total Akt, phospho- and total MAPK, GAPDH (Cell Signaling); β-actin (Sigma Aldrich). KRAS activity was assessed by using the RAS activation kit (Upstate). Visualization was accomplished by using horseradish peroxidase-linked anti-rabbit and anti-mouse secondary antibodies (Cell Signaling) and ECL-Plus blotting substrate detection kit on a Typhoon phospho- and fluorescent imaging system (GE Biosciences).

**Immunohistochemistry.** Immunohistochemistry was performed based on previously described methods [142]. The AE1/AE3 human cytokeratin 7 and cytokeratin 20 antibody (Dako), Smad4 (Santa Cruz), cleaved caspase-3 (Cell Signaling), MIB1/Ki67 (Dako), and cleaved PARP (Abcam).
**Microarray analysis.** The genomic and expression changes caused by TGF-β resistance and KRAS$^{G12V}$ expression were evaluated using the Illumina HumanOmni5Quad and HumanHT-12 v4 array, respectively (Illumina, San Diego, CA). The data were normalized using log$_2$-transformation and quantile normalization. Moderated paired t-tests were used to compare samples and controls. Common differences in fold changes that were ≥ 2-fold were included in our analyses carried out using SAS v9.2. GO term and KEGG pathway analysis were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 and GSEA from the Broad Institute.

### 4.4 Results

#### 4.4.1 TGF-β resistant (TβR) cell line.

The TβR cell line was generated by stepwise culture of the H6c7 cells in increasing concentrations of TGF-β until a cell line emerged that was completely insensitive to the growth inhibitory properties of TGF-β. QPCR was performed to determine which component of the canonical signalling pathway was disrupted. There was a reduction in expression of TGFB$\beta$2R by 45% and Smad4 expression was absent compared to the H6c7 cell line (Figure 4.1A).
Figure 4.1. **Characterisation of the TβR cell line.** (A) TGF-β receptors and Smad expression were assessed by Q-PCR. The H6c7 cell line served as a control. (B) Methylation specific PCR was performed on bisulfite treated gDNA isolated from H6c7 and TβR cells. (C) H6c7 and TβR cells were treated with 5-azacytidine for 5 days and Smad4 expression was assessed by Q-PCR. Untreated TβR cells served as the control. (D) H6c7 and TβR cells were treated with 5-azacytidine for 10 days and Smad4 expression was assessed by Western blotting. (E) Sequencing results of Smad4 exon 1 demonstrate a nonsense mutation L23* in the TβR cell line compared to the H6c7 control.

To determine the mechanism behind the lack of Smad4 expression we examined Smad4 copy number, promoter methylation, and mutational status. There was a 29% decrease in the number of copies of Smad4 in the TβR cell line compared to H6c7 cells as assessed by QPCR. A 21% decrease in chromosome 18 copy number was confirmed by fluorescence in situ hybridization (FISH). Despite the decrease in the number of Smad4 and chromosome 18 copies, this does not fully explain the loss in gene expression. Methylation specific PCR (MSP) was
performed on bisulfite treated gDNA isolated from the H6c7 and TβR cell line revealed that the Smad4 promoter is methylated in the TβR cell line (Figure 4.1B). Treatment of the TβR cell line with the methyltransferase inhibitor, 5-azacytidine, partly restored Smad4 expression (Figure 4.1C-D). Aberrant promoter methylation accounts for the loss of expression for one copy of Smad4, but does not fully account for the loss in expression. Genomic sequencing of the Smad4 gene revealed a nonsense mutation at codon 23 in exon 1. A T to A transition caused an amino acid change from leucine (TTG) to a stop codon (TAG) (Figure 4.1E). Sequencing of the other ten exons did not uncover any other sites of mutation. Thus, the selective pressure of TGF-β stimulation led to Smad4 silencing through the loss of Smad4 gene copies, methylation of the promoter, and mutation of the Smad4 gene.

4.4.2 Expression of KRAS$^{G12V}$ in the TβR cell line.

Stable KRAS$^{G12V}$ expression in the TβR cell line was achieved using an amphotropic retrovirus. KRAS gene expression was 10-fold higher than in the TβR or in the empty-vector control cell line, TβR pBp (Figure 4.2A). KRAS activation assays indicated that KRAS was active in the TβR KRAS cell line and at minimal levels in the control and parental cell lines (Figure 4.2B). However, enhanced MAPK and AKT activation were not observed compared to TβR pBp.
Figure 4.2. **KRAS<sup>G12V</sup> expression in the TβR cell line.** (A) KRAS mRNA expression in H6c7, TβR, TβR pBp, and TβR KRAS cell lines. (B) Western blots of activated RAS, phospho- and total AKT, and phospho- and total MAPK. GAPDH was used as a loading control. (C) TGF-β receptors and Smad expression were assessed by Q-PCR. The H6c7 cell line served as a control. (D) Western blots of phospho- and total Smad2/3, and Smad4. GAPDH was used as a loading control. The mRNA expression levels of (E) PAI-1 and (F) SMAD7 after 48 hours of stimulation with TGF-β in the H6c7 and TβR cell lines. Growth curves of H6c7, TβR, TβR pBp, and TβR KRAS without (G) and with TGF-β.
TGF-β (H). (I) DNA histograms before and after TGF-β treatment. (J) Invasion assays on membranes coated with Matrigel (n=6) incubated without and with TGF-β.

4.4.3 **TGF-β responsiveness.**

QPCR was performed to determine if KRAS\(^{G12V}\) expression had any effect on the expression of the canonical TGF-β signaling pathway components. Copy number analysis revealed a 49% decrease in the number of Smad4 copies in the TβR KRAS cell line. Furthermore, sequencing of exon 1 did not display any sites of mutation. A 74% decrease in the expression of TGFBRI was observed, but had no effect on the downstream phosphorylation of Smad2 and -3 (Figure 4.2C, D). PAI-1 and Smad7 failed to be induced in the TβR cell lines after TGF-β stimulation (Figure 4.2E, F). Smad4 loss and/or KRAS\(^{G12V}\) expression did not alter cellular proliferation, nor were there any responses to TGF-β treatment (Figure 4.2G-I). Treating the parental H6c7 cell line with TGF-β abrogated cell growth and promoted G1 arrest (p<0.001).

4.4.4 **TβR cell lines demonstrate increased invasiveness.**

The TβR cell line had 5-fold higher invasive ability through Matrigel coated Boyden chambers compared to Hc67 cells (p<0.05; Figure 4.2J). KRAS\(^{G12V}\) expression did not further contribute to invasive potential. TGF-β is known to potently induce invasion in epithelial cells. There was a near 6-fold increase in the number of invasive H6c7 cells after TGF-β stimulation compared to the untreated control. However, TGF-β stimulation of the TβR cell lines did not confer the same effect as seen in the H6c7 cell line.
4.4.5 *Tumorigenic ability of the TβR-KRAS cell line.*

Subcutaneous implantation of the TβR and TβR pBp cell lines failed to form tumours in NOD SCID mice (Table 4.2). To assess the tumorigenic ability, the TβR KRAS cell line was implanted subcutaneously and orthotopically into NOD-SCID mice. Tumours developed with complete penetrance in mice injected with the TβR KRAS cell line (Figure 4.3A). Histology from subcutaneous and orthotopic tumours derived from TβR KRAS cells revealed poorly differentiated carcinoma (Figure 4.3A). Regional metastases were identified in the liver and spleen of 15% and 77% of animals, respectively (Figure 4.3B-C, Table 4.2.). Examination of the regional metastases revealed similar histology to the primary orthotopic tumour.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Subcutaneous</th>
<th>Orthotopic</th>
<th>Regional Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. animals</td>
<td>Tumour weight (g)</td>
<td>Tumour weight (g)</td>
</tr>
<tr>
<td>H6c7</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TβR</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TβR pBp</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TβR KRAS</td>
<td>20/20</td>
<td>0.92 ± 0.04</td>
<td>1.21 ± 0.03</td>
</tr>
<tr>
<td>TβR KRAS EV</td>
<td>10/10</td>
<td>0.75 ± 0.06*</td>
<td>1.29 ± 0.14*</td>
</tr>
<tr>
<td>TβR KRAS Smad4</td>
<td>9/10</td>
<td>0.39 ± 0.08</td>
<td>0.91 ± 0.09</td>
</tr>
</tbody>
</table>

Table 4.2. The effect of KRAS and Smad4 expression on the tumourigenicity of the TβR cell lines. The incidence of tumour and metastasis formation was assessed in NOD SCID mice after subcutaneous or orthotopic implantation of two million cells. *N/A* indicates not assessed; *No.*, number; the numbers in parentheses represent the percentage of animals, and *wt*, weight. *denotes significance of *p*<0.05 by Fisher’s exact test for metastatic spread or log-rank for survival; data are presented as mean ± SEM.
Figure 4.3. **Implantation of TβR KRAS forms tumours in NOD-SCID mice.** (A) Tumour growth curve after subcutaneous implantation of the TβR, TβR pBp, and TβR KRAS cell lines into NOD-SCID mice (n=20). Representative H&E sections of xenografts derived from (B) subcutaneous and (C) orthotopic implantation. (D, E) Metastases were found in the liver and spleen after orthotopic implantation of the TβR KRAS cell line.

### 4.4.6 Smad4 expression in the TβR-KRAS cell line.

Smad4 has been previously demonstrated to be a potent tumour suppressor [107-109]. Since KRAS oncogene expression transformed the TβR cell line we wanted to determine if Smad4 expression would suppress tumourigenicity. Stable Smad4 expression in the TβR cell line was achieved via lentiviral construct fused with a GFP tag. Smad4 mRNA expression ranged between 2-4 fold higher in the TβR, TβR-pBp, and TβR-KRAS cell lines compared to the H6c7 cell line, and this restored expression of the type I and type II receptors (Figure 4.4A). Smad4
protein expression corresponded similarly with mRNA expression levels (Figure 4.4B). TGF-β stimulation of the Smad4 expressing TβR cell lines revealed phosphorylation of Smad2 and Smad3 similar to the controls.

Figure 4.4 Smad4 restoration in the TβR cell line. (A) TGF-β receptors and Smad expression were assessed by Q-PCR. The H6c7 cell line served as a control. (B) Western blots of phospho- and total
Smad2/3, and Smad4. GAPDH was used as a loading control. Growth curves of H6c7, TβR, TβR pBp, and TβR KRAS after restoration of Smad4 without (C) and with TGF-β (D). The mRNA expression levels of (E) DNA histograms before and after TGF-β treatment. (F) PAI-1 and (G) SMAD7 after 48 hours of stimulation with TGF-β in the H6c7 and TβR cell lines after forced expression of Smad4. (H) The effect of restoring Smad4 was assessed using invasion assays on membranes coated with Matrigel (n=6) incubated without and with TGF-β.

Smad4 restoration in the TβR cell lines did not alter cellular proliferation as compared to controls (Figure 4.4C). However, it did sensitise the TβR cell lines to the cytostatic properties of TGF-β (Figure 4.4D, E). TGF-β responsive genes PAI-1 and Smad7 were induced after 48 hours of TGF-β stimulation in the Smad4 containing TβR cell lines (Figure 4.4F, G). Restoring Smad4 attenuated the invasive phenotype of the TβR and TβR pBp cell lines, however this effect was not observed in combination with KRAS<sup>G12V</sup> expression (Figure 4.4H). TGF-β stimulation of the Smad4 expressing TβR and TβR pBp cell lines significantly increased the number of invasive cells compared to the untreated controls (p<0.05).

4.4.7 Smad4 restoration delays tumour growth.

We next wanted to determine if Smad4 expression is sufficient to mitigate tumour growth in the TβR KRAS cell line. Palpable masses were detected eight days after subcutaneous implantation of the TβR KRAS EV cells in NOD SCID mice (Figure 4.5A). Whereas, restoring Smad4 expression significantly delayed tumour growth (p<0.0001) and palpable masses were first detected 41 days after implantation. Western blotting and immunohistochemistry confirmed Smad4 expression (Figure 4.5B).
Figure 4.5. **Restoring Smad4 reduces tumour growth and spread.** (A) Growth curves of TβR pBp EV, TβR pBp Smad4, TβR KRAS EV, TβR KRAS Smad4. (B) Western blots of Smad4 and KRAS. β-actin was used as a loading control. (C) Representative histological images of xenografts formed by TβR KRAS EV and – Smad4 cells after H&E, and immunostaining for Smad4, cleaved caspase-3, and Ki67. Scale bars represent 50 μm. (D) The liver and spleens after orthotopic implantation of the TβR KRAS cell line with and without Smad4 overexpression. (E) Quantification of Ki67, cleaved caspase-3, and cleaved PARP positive pixels of the TβR KRAS EV and – Smad4 xenografts. Data is represented by mean ± SEM.
Consistently, orthotopic implantation of the TβR KRAS Smad4 cells in the pancreata of NOD SCID mice displayed longer latency in the detection of palpable abdominal masses and deteriorating condition. Restoring Smad4 expression attenuated metastatic spread to the kidneys and the ubiquitous spread of metastases to the spleen (Figure 4.5D; Table 4.2; p<0.05). We found a significant increase in cleaved caspase-3 in the Smad4 expressing xenograft samples (Figure 4C, E; p=0.007). Additionally, Smad4 expression in the xenografts was associated with an increase in cleaved PARP (p=0.047). No differences were noted in Ki67 staining following Smad4 restoration, thus suggesting that reinstating Smad4 expression promotes apoptosis and does not affect proliferation.

### 4.4.8 Genomic profiling of TβR, TβR-KRAS, and TβR-KRAS-Smad4.

Genomic instability is a common feature in human malignancy. Genomic profiling was performed to ascertain changes that occurred after acquiring TGF-β resistance and KRAS expression. The aCGH profile of the H6c7 cell line confirmed previously published findings of losses on 3p, 10p12, and 13q14, and gain on 20 [74]. The TβR cell lines displayed further losses of 10q25.2-26.3, 11q14.3, 19q13.33, and 22q13.2-13.33, and gains on 2p22.3, 5p15.33-13.2, and 13q31.1-34, and copy neutral loss of heterozygosity on chromosome 18 compared to the parental H6c7 cell line (Figure 4.6). The expression of KRAS resulted in further gains on 3q, 4p, 7q31.32-36.3, 12q21.2, 12q21.32, and 14q23.1-32.33, and loss on 18.
Figure 4.6. The distribution of the acquired gains and losses due to TGF-β resistance and KRAS\(^{G12V}\) expression. The blue lines represent changes found in the TβR cell lines and purple lines represent changes found after KRAS\(^{G12V}\) expression. Genes in red letters represent gain in copy number, whereas genes in green represent loss in copy number.

Examining gene alterations in the TβR cell lines produced a list of known oncogenes and tumour suppressors. Acquiring TGF-β resistance resulted in copy number gains of oncogenes TERT, ID1, and SRC (Appendix Table 3). CNV analysis revealed a hemizygous deletion on chromosome 3 which accounts for the reduced TGFBR2 expression and a copy neutral loss of
heterozygosity on chromosome 18 where Smad4 lies. Common losses on tumour suppressor genes VHL, EP300, RB1, BRCA2, and BUB3 were observed in the TβR cell lines (Appendix Table 4). KRAS$^{G12V}$ expression led to the copy number gain in genes including AKT1, BRAF, PI3KCA, FOS, KIT, SMO, HIF1A, and PDGFR2; and copy number losses in Smad4, Smad2, and DCC.

**Figure 4.7. Pathway analysis of the copy number gains and losses due to TGF-β resistance and KRAS$^{G12V}$ expression in the H6c7 cell line.** Summary of the signaling pathways and processes altered after gaining TGF-β resistance and KRAS$^{G12V}$ expression. A complete list of genes associated with these pathways and processes are listed in the Appendix in Tables 3 and 4.

To assess how these genomic changes affected the TβR cell lines, pathway and functional analyses were performed on the genes that were mapped to the regions of gains and losses absent in the H6c7 cell line (Figure 4.7). Acquisition of TGF-β resistance amplified genes involved in G-protein signaling and adhesion, and genomic losses in cell cycle regulation, chemotaxis, and NF-κB signaling. KRAS expression led to losses which altered the Wnt signaling, and G-protein signaling, and gains in wound healing, secretion, drug metabolism, and negative regulation of apoptosis.
4.4.9 *Global expression profiling of TβR, TβR-KRAS, and TβR-KRAS-Smad4.*

Pathway and functional analyses were performed on gene alterations due to TGF-β resistance. These analyses revealed losses in TGF-β signaling pathway and gains in cell cycle, adhesion, angiogenesis, and cell-cell signaling (Figure 4.8, Appendix Table 5). KRAS\(^{G12V}\) expression resulted in losses in Wnt Signaling, and gains in angiogenesis, cell cycle, wound healing, and negative regulation of cell death. Smad4 restoration had little effect on the genome as the majority of changes were observed after KRAS expression. We also examined gene expression changes after acquiring TGF-β resistance and KRAS\(^{G12V}\) expression. Gaining TGF-β resistance resulted in downregulation of genes involved in extracellular matrix (ECM) and pathways in cancer (Appendix Table 6). KRAS\(^{G12V}\) expression induced expression of processes involved with JAK/STAT signaling, angiogenesis, and motility, and downregulated genes involved in non-canonical hedgehog signaling, adhesion, and ECM. Expressing Smad4 promoted gene expression in ECM-receptor interaction, ECM, and actin cytoskeleton regulation, and downregulated MAPK signaling pathway.

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**Figure 4.8. Pathway analysis of the gene expression changes due to TGF-β resistance and KRAS\(^{G12V}\) expression in the H6c7 cell line.** Summary of the signaling pathways and processes altered after gaining TGF-β resistance and KRAS\(^{G12V}\) expression. A complete list of genes associated with these pathways and processes are listed in the Appendix in Tables 5 and 6.
Table 4.3. Cell line phenotypes of the TβR cell lines in comparison to H6c7.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumourigenicity</th>
<th>Metastasis</th>
<th>Invasion</th>
<th>Proliferation</th>
<th>TGF-β sensitive</th>
</tr>
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<tbody>
<tr>
<td>H6c7</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TβR</td>
<td>0/5</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TβR pBp</td>
<td>0/5</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TβR KRAS</td>
<td>20/20</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TβR pBp EV</td>
<td>0/5</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TβR pBp Smad4</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TβR KRAS EV</td>
<td>26/26</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>TβR KRAS Smad4</td>
<td>28/29</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

4.5 Discussion

The results of our study indicate that in an immortalised human pancreatic epithelial duct cell line that Smad4 loss and KRAS$^{G12V}$ expression are sufficient for tumourigenic transformation. KRAS$^{G12V}$ expression alone is inadequate for neoplastic transformation of the immortalised H6c7 cell line. Smad4 absence is obligatory for KRAS mediated malignant transformation to occur, and its restoration in the TβR cell line significantly represses *in vivo* tumour growth by promoting cell death and suppressing invasion. Thus, Smad4 serves as a restriction point for the transformation of normal human pancreatic duct cells.

In PDAC there are frequent disruptions in the TGF-β signalling pathway. Loss of heterozygosity of 18q is a common event that occurs in over 90% of pancreatic carcinomas. Biallelic loss of Smad4 is found 55% of cases in pancreatic cancer. The loss of the second allele has been attributed to deletion in 35% and mutation in 20% [45]. Smad4 loss in the TβR cell line was caused by the step-wise selection in increasing concentrations of TGF-β. The mechanism for this loss of expression can be attributed to copy number loss, methylation of the Smad4 promoter, and a nonsense mutation in the Mad Homology (MH) 1 domain. Compared to the
parental H6c7 cell line, there was a reduction in TGFBR2 expression by 45% and Smad4 expression was nearly absent. CNV analysis revealed a hemizygous deletion on chromosome 3 which accounts for the reduced TGFBR2 expression, and a copy neutral loss of heterozygosity on chromosome 18 where Smad4 lies. Copy number analysis by QPCR and chromosome 18 FISH found similar losses in Smad4 copy number. Hypermethylation of the Smad4 promoter is not associated with pancreatic cancer [168]. Though, it has been noted in prostate cancer [169], Barretts oesophagus [170], and gastric cancer [171]. In PDAC, mutations have been found to be localised within the MH1 and -2 domains [172]. Mutations in the MH2 domain render Smad4 unable to heterooligomerise with R-Smads [173]. Whereas, mutations in the MH1 domain increase autoinhibition of Smads by stabilizing the interaction between MH1 and MH2, and enhance degradation by the ubiquitin-proteasome pathway [174,175]. KRAS$^{G12V}$ expression promoted copy number loss of chromosome 18 loss which reduced the number of Smad4 copies by half. Together these data indicates that there is a loss of one copy in Smad4 in half of the TβR population, and that expression loss of the remaining Smad4 copies can be attributed promoter methylation and a nonsense mutation. Oncogenic KRAS expression furthered TGF-β insensitivity through the loss of heterozygosity of chromosome 18 which mirrors what is observed in patients.

KRAS oncogene expression promotes malignant transformation in the TβR cell line. Other pancreatic epithelial cell models have also examined the transformative ability of KRAS. A human pancreatic Nestin positive epithelial (HPNE) cell line has been demonstrated to be transformed by KRAS after immortalization with hTERT, and introduction of E6, E7, and small t antigen [86]. Bovine pancreatic duct epithelial cells immortalised with SV40 large T antigen were not completely transformed after KRAS$^{G12V}$ expression [72]. Incomplete transformation by KRAS$^{G12V}$ was also observed in rat pancreatic epithelial cells [70]. Thus, KRAS expression
alone is insufficient for complete transformation of immortalized pancreatic duct cells. Additional events are required besides KRAS expression for tumourigenic transformation of pancreatic duct cells, thus Smad4 loss was essential for KRAS induced transformation of the H6c7 cell line.

Restoring Smad4 expression reversed the invasive phenotype of the TβR cell lines, and reduced tumour growth in the TβR-KRAS cell line. Similar work performed in other pancreatic cancer cell lines such as BxPC3 [107,108], Hs766T [106], CFPAC1, and Capan1 [110] also demonstrate decreased invasion and tumour growth. Analogous findings have also been observed in ovarian [109], breast [112], and colon [176] cancer cell lines after reconstituting Smad4 expression. Smad4 expression promoted apoptosis in the TβR KRAS xenografts independent of p53 and RB, since both proteins were inactivated after immortalizing the H6c7 cell line. Comparable work performed in the breast cancer cell line, MDA-MB-468, identified that expression of Smad4 induced apoptosis in the absence of p53 and RB [177]. Similarly, restoring TGF-β sensitivity by expressing TGFBR2 in PDAC cell line, MiaPaCA-2, enhances susceptibility to apoptosis by upregulating pro-apoptotic Bax [178]. These studies validate our findings that restoring TGF-β sensitivity critically reverses some of the tumorigenic characteristics of the TβR KRAS cell line.

The development of genomic instability is a hallmark of cancer. The acquisition of amplifications and deletions facilitate the genetic changes necessary for malignant transformation. Acquiring TGF-β resistance caused copy number losses in pathways involved in TGF-β signaling. Perturbing the TGF-β signaling pathway was a necessary adaptation to foster survival when incubated with a cytostatic cytokine. Analysis of the gene expression alterations between the TβR and H6c7 cell lines revealed enrichment in cell motility and decreases in ECM which is congruent with the phenotypic differences in invasive ability between these two cell
Further examination of these genes may illuminate how Smad4 loss promotes cellular invasion. KRAS$^{G12V}$ caused the greatest degree of genomic alterations which promoted cell motility and MAPK signaling, and gene expression changes enriched for angiogenesis, Wnt pathway, and JAK-STAT signaling. Wnt signaling has been identified to promote survival during extravation and metastatic dissemination [179]. Restoring Smad4 caused DNA copy number gains and gene expression changes that promoted ECM restructuring. Though the majority of genomic changes occurred after introduction of the KRAS oncogene, reinstating Smad4 expression re-established TGF-β sensitivity and induction ECM related genes.

The exomic sequencing of 24 invasive PDACs identified 12 core signaling pathways that are perturbed in pancreatic cancer [180]. We observed alterations in the TGF-β, KRAS, and Wnt signaling pathways; cell adhesion; invasion/motility; and apoptosis after analysing the genomic and gene expression changes. The changes seen in these core signaling pathways overlap with this model and validate these patient data [180]. These alterations have yielded promising insights into the requirements for tumorigenic transformation of the H6c7 cell line. Further investigations into the identified genes may shed additional insight into the pathogenesis of this fatal cancer.

This study has chronicled the evolution from normal pancreatic duct epithelial cell to tumour cell under selective pressure and oncogene expression. In summary, we have demonstrated that significant Smad4 downregulation in a near normal pancreatic duct epithelial cell line with inactivated Rb and p53 is insufficient for KRAS mediated malignant transformation. Rather, Smad4 loss is obligatory to enhance KRAS driven tumorigenicity. Smad4 expression restores TGF-β sensitivity and represses tumour development through promotion of apoptosis, and reduces metastatic dissemination. The TβR cell line provides a model for examining how Smad4 deficiency promotes pancreatic carcinogenesis. Usage of the
H6c7 cell line as a model of normal human pancreatic duct cells has provided a crucial platform to study the mechanistic roles of oncogenic KRAS and Smad4 loss.
Chapter 5

Discussion and future directions

5.1 Discussion

Pancreatic cancer is a disease that is characterised by the step-wise accumulation of genetic alterations in KRAS, p16, p53, and Smad4 which cooperate in the malignant transformation of duct epithelial cells [15]. The near ubiquitous activation of KRAS in patients, and its ability to initiate and maintain disease in mouse models indicate its importance in PDAC [49,52]. Furthermore, targeted loss of function of these tumour suppressor genes in combination with KRAS accelerated pancreatic tumour development which recapitulates human disease. The majority of pancreatic cancers occur within the duct, however these murine models have targeted KRAS expression in the developing pancreas, acinar, and endocrine cells as there are no duct specific models currently [49,58]. Extrapolating what has been learnt from these murine pancreatic cancer models, the future identification of duct specific promoters may truly reiterate human disease.

Despite the wealth of information gained from animal models, species differences exist in neoplastic transformation of murine and human cells [82]. In non-human pancreatic duct models, mutant KRAS expression fails to transform bovine and murine PDEC cells [70,71,88]. Neoplastic transformation of mPDECs require the forced expression of Twist to inhibit induction of p16\(^{INK4A}\) [71]. The human pancreatic Nestin expressing cell line, HPNE, was immortalised using E6 and E7 and malignant transformation was caused by the co-expression of KRAS\(^{G12D}\) and small T (st) antigen from SV40. This model of KRAS-driven transformation depends on st
antigen to inactivate the tumour suppressor serine-threonine protein phosphatase 2A [86]. In this work, the immortalised H6c7 cell model was used to examine pancreatic carcinogenesis in a human context and in the cell type of origin. The employment of an ecotropic retroviral system introduced tetraploidy in the H6c7 cell line that likely permitted partial transformation with KRAS<sup>G12V</sup> alone [84]. KRAS<sup>G12V</sup> expression alone in the absence of genomic instability does not transform the H6c7 cell line. Accordingly, these models and this work demonstrate that oncogenic KRAS expression requires further genetic alterations to transform immortalized cells.

5.1.1 The role of LCN2 in pancreatic carcinogenesis

The introduction of KRAS<sup>G12V</sup> induces LCN2 expression in the H6c7 cell line [84]. LCN2 expression has been observed in numerous cancer types including breast, lung, ovary, thyroid, esophageal, and PDAC [118-122]. Enhanced LCN2 expression was identified in the H6c7 eco-KRAS and H6c7 Kras Tumour (KrT) cell line compared to the H6c7 cell line. The majority of PDAC cell lines express elevated LCN2 levels. In addition, LCN2 immunoreactivity was associated with high-grade PanIN lesions and pancreatic cancer tissue. Together these data indicate that ectopic LCN2 expression is a potential biomarker for pancreatic cancer.

Using multiple PDAC models clearly demonstrated that LCN2 promotes invasion through enhancing MMP-9 activity, tumourigenicity, and survival. LCN2 was first identified in neutrophils attached to MMP-9 as a homodimer which enzymatically activates and sustains MMP-9 activity [123,124]. The LCN2-MMP-9 complex has been associated with enhanced invasion and increased metastatic potential in breast cancer [122,125,126], esophageal [121], and gastric cancers [127]. LCN2 depletion in colon cancer [143], gastric cancer [128], and breast cancer [125,129] cell lines attenuates cellular invasion by diminishing MMP-9 activity. Together, this work supports findings in other cancer types and further exploration is required to
determine if LCN2 affects metastasis in the high and low LCN2 expressing pancreatic cancer cell line models.

In addition to its role in promoting MMP-9 activity, LCN2 has also been reported to promote tumourigenesis. In this study, LCN2 expression was suppressed using shRNA in two PDAC cell lines, BxPC3 and HPAF-II, and overexpressed in the PANC-1 cell line. LCN2 suppression increased tumour growth latency in mice bearing the BxPC3 and HPAF-II LCN2KD2 xenografts. Similarly, studies in breast cancer have demonstrated that LCN2 accelerates tumour growth [122,125,129,145] and metastasis [125,129,145]. Nevertheless, how LCN2 modulates tumour growth has not been fully elucidated. No differences were ascertained in Ki67 immunostaining or caspase-3 cleavage between low and high expressing LCN2 xenografts. In sum, these data indicate that LCN2 does not affect proliferation or survival in the absence of a cytotoxin. LCN2 has been implicated in angiogenesis by regulating VEGF expression [134]. Examination of CD31 positive blood vessels in the BxPC3 and PANC1 xenografts revealed that LCN2 is associated with increased vascularity. Interestingly, a recent mouse study suggests that LCN2 is upregulated during hypoxia and that HIF1A may act as an intermediary in controlling LCN2 expression [181]. Gene expression analysis identified HIF1A as one of the significantly upregulated genes. HIF1A and VEGF gene expression were elevated in LCN2 expressing xenografts, thus validating the microarray and histology. Together, these data suggest that LCN2 contributes to tumour growth by enhancing tumour vasculature. However, additional studies are required to determine how LCN2 regulates HIF1A and VEGF expression, if HIF1A regulates LCN2 in the human model, and if LCN2 is upregulated in the response to tumour hypoxia.

Additionally, LCN2 has also been reported to promote tumourigenesis by acting as an anti-apoptotic protein. Several studies have reported that the expression of LCN2 in lung, breast
[130], and thyroid [120] cancer cell lines blocks the induction of apoptosis \textit{in vitro}.

Transcriptional profiling revealed that LCN2 enhanced expression of genes that are involved in cell survival, cell cycle progression, and adhesion, and downregulated a set of pro-apoptotic genes. This is the first published study to examine the relationship between chemotherapeutic sensitivity and LCN2 expression in PDAC \textit{in vivo}. In agreement with previous findings, LCN2 expression decreases sensitivity of PDAC cells to gemcitabine \textit{in vitro}. Furthermore, depleting LCN2 in the Gemcitabine resistant BxPC3 xenografts sensitizes the model to Gemcitabine and increased caspase-3 cleavage. Gemcitabine sensitive PANC1 xenografts were partially conferred resistance to chemotherapy, however this effect was not significant. Testing additional PDAC cell line xenograft models should be performed to support the relationship between LCN2 and drug resistance. Recently, a breast cancer report has associated LCN2 expression with poorer disease free survival and poor response to neoadjuvant chemotherapy [182]. Though PDAC is a lethal disease and LCN2 was found to be commonly expressed in PDAC, further examination of LCN2 expression and survival and treatment may act as a predictive marker to treatment efficacy.

In contrast to the numerous studies that have indicated that LCN2 supports invasion and survival, there have been several reports showing contradictory findings. LCN2 has been shown to be secreted in leukemic mouse models expressing BCR-ABL which induces apoptosis in normal hematopoietic cells and facilitates leukemic cell dissemination [135]. In HRAS transformed mouse mammary tumour cells, LCN2 abrogates Raf activation thereby suppressing EMT [132] and VEGF expression [150]. LCN2 overexpression reduces xenograft growth and metastasis in the colon cancer cell line, KM12SM, [133] and PDAC cell line, MiaPaCa-2, [134]. To reconcile the differences seen between PANC-1 and MiaPaCa-2 models after LCN2 expression, LCN2 should be restored in the MiaPaCa-2 cell line, and the \textit{in vitro} and \textit{in vivo}
studies should be repeated. This discrepancy in findings after LCN2 was expressed in the MiaPaCa-2 may be due to cell line contamination, the LCN2 expression construct employed, or experimental design. Further exploration with other pancreatic cancer or other tumour cell line models may reconcile the differences seen between these investigations.

5.1.1.1 **Future directions: LCN2 in drug resistance and iron regulation**

LCN2 has been implicated to be an anti-apoptotic protein, it would be advantageous to investigate if LCN2 promotes resistance to other chemotherapeutic agents. As presented in Chapter 2, knocking down LCN2 expression in the BxPC3 and HPAF cell lines enhanced sensitivity to gemcitabine *in vitro*. *In vivo*, treating tumour bearing mice implanted with BxPC3-LCN2KD2 cells with gemcitabine greatly reduced tumour growth compared to vehicle treated mice. Gemcitabine can be administered as a single agent, or in combination with radiotherapy or other chemotherapeutic agents. The combination of gemcitabine with other cytotoxic agents such as cisplatin [183], oxaliplatin [184], and 5-flurouracil [185] have been shown to increase median survival. The only combination with gemcitabine that has demonstrated a significant increase in survival was the addition of erlotinib, an EGFR tyrosine kinase inhibitor [186].

To assess how LCN2 expression affects chemosensitivity in pancreatic cancer, cells were treated with cisplatin or erlotinib. Cisplatin is a platinum containing chemotherapeutic drug that halts DNA replication by crosslinking DNA. LCN2 expression had no impact on the sensitivity of BxPC3 and PANC-1 cells (Figure 5.1A). However, LCN2 depletion further sensitizes the HPAF-II cell line to cisplatin (p=0.02). EGFR activation has been shown to induce LCN2 expression [181] and EGFR expression has been found in 69% of pancreatic cancers [187]. Erlotinib is an EGFR kinase inhibitor which binds to the ATP binding site of the receptor in a reversible fashion [12]. Erlotinib treatment sensitised the low LCN2 expressing BxPC3 and
HPAF-II cell lines by 4% and 13%, respectively (p<0.05; Figure 5.1B). Whereas, LCN2 expression promoted survival by 6% after 72 hours of erlotinib treatment (p=0.007). These preliminary data encouragingly demonstrate that LCN2 depletion can sensitize cells to erlotonib. The results of these preliminary analyses, as well as survival analysis from the gemcitabine and erlotinib study may provide additional evidence for stratifying patient treatment based on LCN2 expression [186].

Figure 5.1. The role of LCN2 in drug resistance and iron regulation. PI exclusion assays for cell death after 72 hours treatment with (A) cisplatin, (B) Erlotinib, and (C) DFO and gemcitabine on the BxPC3, HPAF-II, and PANC1 cell lines. (* denotes significant differences between the test and control samples, † denotes significant differences between gemcitabine and gemcitabine in combination with DFO; p<0.05).
Another facet of LCN2 function is its ability to facilitate iron transport into cells. Regulation of iron transport has been previously implicated to be anti-apoptotic [120,188]. Because of this, we wanted to determine if the iron chelator, DFO, could also provide an additive benefit to gemcitabine treatment. Use of DFO had no effect on gemcitabine sensitivity in the control BxPC3 and HPAF-II cell lines. However, concomitant treatment of DFO and gemcitabine in LCN2 depleted BxPC3 and HPAF-II for 72 hours resulted in a 13.5 and 6.8% increase in the number of apoptotic cells, respectively (p<0.001; Figure 5.1C). Similarly, PANC-1 cells treated with this combination therapy increased its sensitivity by 20%. In comparison, stable LCN2 expression in PANC-1 cells did not exhibit any changes in survival. It would be advantageous to further examine this drug combination in xenograft models. DFO is currently used in the clinic for iron overload disorders and has had limited success as a cancer treatment. Hydrophobic iron chelators have demonstrated increased anti-tumoral activity over DFO, and low toxicity which makes it an attractive drug to use in combination with chemotherapy [189]. Usage of hydrophobic iron chelators in conjunction with gemcitabine may potentiate further cytotoxicity in vivo. Furthermore, preclinical studies can be performed using patient derived xenograft models grouped based on LCN2 expression. These models can be treated with single and combinations of erlotinib, iron chelator, and gemcitabine to determine if LCN2 can be used as a predictive biomarker. Elevated LCN2 expression in various cancer types makes it an attractive biomarker for diagnosis, prognostication, and therapeutic targeting.

Lastly, since LCN2 has been demonstrated to be commonly overexpressed in PDAC the employment of a monoclonal antibody may increase chemosensitivity. Targeted therapies in pancreatic cancer have shown limited efficacy in the clinic [190]. Therefore, an attractive strategy in the treatment of pancreatic cancer may be to combine gemcitabine treatment and attenuate LCN2 expression. The recent development of a monoclonal antibody against murine
LCN2 was shown to reduce tumour growth and metastatic spread of a mouse mammary tumour model [125]. It is interesting to speculate that addition of a neutralizing antibody targeted against LCN2 may sensitize tumours to chemotherapy and attenuate metastasis by interfering with its association with MMP-9.

Evidence from several studies has indicated that LCN2 promotes tumourigenicity through its role in survival and invasion. However, how LCN2 promotes survival has not been fully elucidated. LCN2 has been implicated in preventing the intrinsic apoptotic program by inhibiting caspase-9 activation in a thyroid cancer model [120]. It has been demonstrated here that LCN2 expression is associated with reduced caspase-3 cleavage in pancreatic cancer xenograft and cell line models. Employing specific caspase inhibitors and examining upstream caspase activity should further delineate whether LCN2 is involved in the intrinsic or extrinsic apoptotic pathways. Furthermore, expression analysis of pancreatic cancer cell lines with gemcitabine should provide keen insights on which genes are being regulated by LCN2 and are contributing to survival. In sum, elucidating the multifaceted role that LCN2 plays in tumourigenesis will shed light on how to produce effective therapeutic strategies to interfere with LCN2 and its functions.

5.1.2 The role of KRAS and Smad4 in pancreatic carcinogenesis

Malignant transformation requires additional genetic changes beyond mutant KRAS expression. In this study, we examined the role that inactivation of the tumour suppressor Smad4 plays in this transformation. In PDAC, there is a near ubiquitous 18q loss and frequent Smad4 inactivation demonstrates its significance in ductal carcinogenesis [45]. Studies performed in PDAC mouse models have revealed that targeted Smad4 loss in the pancreas does not result in
mPanIN or mPDAC formation. However, its loss in combination with KRAS<sup>G12D</sup> expression can promote the development of cystic precursor lesions and accelerate mPDAC [54, 64]. In this work, Smad4 expression was suppressed by 80% using shRNA. Knocking down Smad4 and KRAS<sup>G12V</sup> expression partially transformed the H6c7 cell line. This incomplete knockdown may have maintained the tumour suppressive function which could act as a restriction point for transformation. Residual Smad4 expression and/or KRAS<sup>G12V</sup> expression may not have introduced the genomic instability necessary to facilitate transformation. High KRAS<sup>G12V</sup> expression or Smad4 knockdown increased the invasiveness of H6c7 cells. Analogous findings have also been reported in the HPNE cell line after KRAS<sup>G12D</sup> expression or Smad4 knockdown [103]. Depleting Smad4 in KRAS<sup>G12D</sup> expressing HPNE cells also promoted colony formation in soft agar, however tumourigenic transformation was not reported [103]. Smad4 loss in PDAC is associated with poorer survival and widespread metastasis [46]. Although differences exist between the HPNE and H6c7 cell lines, similar findings between these studies demonstrate that knocking-down Smad4 promotes an invasive phenotype in pancreatic epithelial cells.

To address the possibility that insufficient Smad4 knockdown was the reason that the H6c7 cells with oncogenic KRAS failed to be transformed, we used the TβR cell line. The TβR cell line was generated from the serial passaging of the H6c7 cell line in increasing concentrations of TGF-β. These cells exhibit Smad4 loss through copy number loss, methylation, and mutation. TGF-β stimulation failed to induce cell cycle arrest and expression of responsive genes in this model system. Despite Smad4 loss, upstream signalling remained intact as evidenced by the phosphorylation of Smad2 and-3 after acute stimulation. Phenotypically, Smad4 absence in the TβR cell lines did not alter cellular proliferation rates, but did displayed increased invasiveness compared to the parental H6c7 cell line. These observations are congruent to what was observed in the H6c7 and HPNE cell lines after knocking
down Smad4 [103]. Smad4 deficiency did not promote pancreatic duct cell carcinogenesis analogous to what was reported in the Smad4 deficient animal models. However, Smad4 loss in combination with targeted KRASG12D expression accelerated tumourigenic transformation in murine pancreata [54,63,64].

KRAS activation in pancreatic cancer has been suggested to be sufficient to cause the initial dysplasia of murine pancreata, however the presence of additional genetic aberrations are required to advance mPDAC [53,54,60]. Similarly, KRAS oncogene expression was insufficient to cause neoplastic transformation of pancreatic cell lines HPNE, bovine and murine PDECs, and H6c7 [70,72,84,86]. Thus, Smad4 inactivation was essential for KRAS induced transformation of the H6c7 cell line. KRASG12V expression transformed the TβR cell line and generated tumour formation in NOD SCID mice with complete penetrance. TGF-β selection and KRASG12V expression introduced additional genomic alterations that permitted malignant transformation. The PDAC pathogenesis is reflected in the H6c7 cell line through the stepwise accumulation of genetic modifications.

Restoring Smad4 tumour suppressor activity in the TβR KRAS cell line reinstated TGF-β sensitivity, diminished the invasiveness of the TβR cell line, significantly delayed in vivo tumour growth, and reduced metastatic spread. Similar findings have also been shown in pancreatic [106-108,110], ovarian [109], breast [112], and colon [176] cancer cell lines. Reinstating Smad4 expression has been found to reduce tumour growth by increasing p15 and p21 expression [110] and decreasing vascularity [176]. The latency in growth of the TβR KRAS Smad4 xenografts was attributed to increased rates of apoptosis. Elevated cleaved caspase-3 and PARP immunoreactivity were found in the Smad4 expressing TβR KRAS xenografts. Smad4 promoted apoptosis in the absence of p53 and Rb, since both proteins were inactivated after immortalising the H6c7 cell line. Similar findings of Smad4 induced apoptosis independent of p53 and Rb have
also been seen in the breast cancer cell line, MDA-MB-468 [177]. Several downstream targets for TGF-β mediated apoptosis have been identified including death associated protein kinase (DAPK), TNF-related apoptosis-inducing ligand (TRAIL), GADD45B, FAS, and Bim [36]. Smad4 loss in the TβR cell lines permitted escape from TGF-β mediated cell cycle arrest and apoptosis in vivo, and enhanced invasion and metastasis. Re-establishing Smad4 expression in the TβR cell lines partially reversed these phenotypes. Thus, this work is in agreement with previous findings that Smad4 is a critical repressor duct carcinogenesis.

5.1.2.1 **Future directions examining the roles of Smad4 and KRAS in pancreatic duct carcinogenesis**

The residual expression of Smad4 in the H6c7-KRAS-S4KD2 cell line may have maintained its tumour suppressive function hindering malignant transformation. Further animal experiments will be required to validate the tumour formation from the engraftment of the H6c7-KRAS-S4KD2 cell line in NOD SCID gamma (NSG) mice. We have recently designed a lentiviral double shRNA expression vector [141]. The introduction of two shRNA sequences targeted against Smad4 may optimize suppression of Smad4 expression and permit KRAS$^{G12V}$ to transform the H6c7 cell line. These Smad4 depleted and TβR cell lines will also be implanted in the NSG strain to validate the findings demonstrating that Smad4 loss is not a tumour initiating event [54,63,64]. Xenotransplantation of the newly Smad4 depleted KRAS$^{G12V}$ expressing H6c7 cell line into NOD SCID or the more immunodeficient NSG strain may also yield increased tumour establishment.

A tumour derived cell line was established from the H6c7-KRAS-S4KD2 xenograft. Similar to the xenograft, the KST1 cell line is depleted of Smad4 expression and maintains KRAS expression. Implantation of this cell line produced tumours in half of the mice assessed. Additional tumour derived cell lines were established from the KST1 xenografts and will be
implanted subcutaneously in mice to evaluate its tumorigenicity. It is conceivable that these additional tumour derived cell lines may generate tumours with higher or complete penetrance. Examining gene and genomic alterations in the tumour and tumour derived cell lines may reveal novel or known genes that were acquired during xenograft formation which may also be involved in pancreatic carcinogenesis. Furthermore, these analyses will shed light on what additional genetic alterations are required for KRAS driven transformation of the H6c7 cell line.

![Graph showing tumour volume over time](image)

**Figure 5.2. Tumourigenicity of H6c7-KRAS-S4KD2 tumour (KST1) derived cell line.** (A) Growth rate of subcutaneous tumours formed by the KST1 cell line. Tumour formation was detected in half of the mice assessed (n=4). (B) Smad4 and KRAS protein expression was examined in the H6c7, H6c7-S4KD2 (S4KD2), H6c7-KRAS-S4KD2 (KS), H6c7-KRAS-S4KD2 xenograft (KSx), KST1, and KST1 xenograft 1 and 2 (KSTx1 and -2). GAPDH was employed as a loading control.
Smad4 deficiency promotes invasion in the H6c7 cell line. Nevertheless, the underlying mechanism of how Smad4 loss mediates invasion needs to be explored. Several reports have found that restoring Smad4 significantly reduces the invasive phenotype [105,107,111]. Gene expression changes were not assessed after Smad4 was knocked down in the H6c7 cell line, however analysis between the H6c7 and TβR cell line have revealed upregulated genes involved in cell migration. Acquisition of TGF-β resistance caused alterations in the gene expression of extracellular matrix components and upregulates cytokines such as IL-6 which have been shown to promote pancreatic tumourigenesis and may account for differences in invasive ability [191]. Additionally, KRAS\textsuperscript{G12V} expression confers increased invasion in the H6c7 and TβR cell line. KRAS\textsuperscript{G12V} expression in the HPNE system relies on the RAF/MEK and PI3K pathways to stimulate migration [87]. Similarly, usage of kinase inhibitors can be employed to determine which downstream pathway KRAS acts through to promote invasion in the H6c7 model. Interestingly, expression of KRAS\textsuperscript{G12V} promoted expression of several Wnt ligands and receptors. A recent report has demonstrated that circulating tumour cells depend on Wnt signaling to promote survival in the blood stream and metastatic dissemination [179]. The expression of these Wnt proteins should be first examined using QPCR to validate the microarray findings. Testing the functional consequences of overexpressing these Wnt pathway components can be determined by QPCR for induction of Wnt target genes, immunoblotting for nuclear localisation of β-catenin, and reporter gene assays. Employment of shRNA and/or Wnt inhibitors can illustrate the dependence of the TβR-KRAS cell line on Wnt signaling for \textit{in vitro} invasion assays and for metastatic dissemination in the orthotopic mouse models.

Pancreatic cancer is characteristically a metastatic disease. Comprehensive genetic analysis has revealed that less than half of the genomic alterations observed in the primary tumour are also evident in the metastases [192,193]. Examination of the metastases found from
the orthotopic implantation of the TβR KRAS and TβR KRAS Smad4 models may reveal genetic evolution from the primary tumour site, and how restoring Smad4 reduces metastatic spread. Restoring Smad4 did not completely repress tumourigenicity of the TβR KRAS cell line. The comprehensive sequencing of 24 invasive pancreatic ductal adenocarcinomas from patients established a set of twelve core pathways that are affected in pancreatic cancer [180]. Examination of genes that are mutated or deleted on chromosome 18 in patients revealed a putative list of potential tumour suppressor genes that may be affected [180]. QPCR validation was performed and it was determined that C18orf45, CCDC68, STARD6, ARHGAP28, LAMA1, RTTN, Smad4, and TCE3B were affected. Since Smad4 re-expression was insufficient to suppress tumour growth then the expression of one of these genes may completely attenuate tumour growth. CCDC68 is commonly lost in colorectal cancer and its function has not yet been determined [194]. Initial experiments have demonstrated a tumour suppressive role in pancreatic cancer. CCDC68 expression alone and in combination with Smad4 will be performed in the TβR KRAS cell line and the resulting cell lines will be assessed for tumour growth in NOD SCID mice.

5.2 Concluding remarks

Pancreatic ductal adenocarcinoma is an aggressive and lethal malignancy. Using genetically defined modifications in the H6c7 cell line we can attempt to understand the contribution of each genetic alteration and identify new pancreatic cancer biomarkers. In the molecular progression model, KRAS is mutated early on and has been implicated to be crucial to maintain pancreatic carcinogenesis. KRAS\textsuperscript{G12V} expression in the H6c7 cell line induces the expression of LCN2 [84]. Currently, CA19-9 is the most commonly used serum biomarker to
diagnose pancreatic cancer [12]. Identification of LCN2 in the urine and blood makes LCN2 an ideal biomarker for prognostication and therapeutic targeting in PDAC. Though there have not been any reports linking Smad4 loss or TGF-β signaling in regulating LCN2 expression. It would be of great interest to explore if there is a relationship between TGF-β and this emerging disease biomarker. Smad4 is lost in half of pancreatic cancers and has been identified in these studies as a restriction point in the transformation of an immortalised normal pancreatic duct epithelial cell line. Though restoring Smad4 did not completely suppress tumourigenesis, it did promote apoptosis and attenuate metastatic dissemination in the orthotopic mouse models. The results of these studies support clinical data that Smad4 status is associated with widespread metastatic disease and poorer survival [46]. In summary, the studies presented here have provided a new platform and additional insights in roles that KRAS and Smad4 play in pancreatic carcinogenesis.
# Appendix

Table 1. LCN2 upregulated genes according to their annotated functions

<table>
<thead>
<tr>
<th>Regulatory process or pathway</th>
<th>Representative genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intracellular non-membrane bound</strong> (n=104; p&lt;0.001)</td>
<td>ABI2, ACTR2, AIF1, ANLN, APOBEC3F, ARID4B, BIN1, BIRC2, BOP1, CASP3, CA5, CDK5RAP2, CEBPG, CKAP2, CSTB, CTNNAL1, CTNNB1, DDX21, DYNLT3, EIF3E, FBXO5, FERMT1, FOXK1, FRMD6, FSCN1, FXR1, GRWD1, GTF2F2, GTPBP4, HILS, HIF1A, HMGA1, HMGB1, HMGB1L1, IGF2BP2, ILK, ING2, INTS6, KIF11, KLK6, KRT14, KRT16, KRT17, KRT6A, KRT6B, KRTAP3-2, LCP1, LOR, LRRCC1, LYAR, MCM4, MPRIP, MYC, MYCBP2, MYL1, NCA, NDRG1, NEB, NMD3, NOP56, NUP133, OASL, ORC6L, PARVB, PFN2, PHF2, PKP1, PLEK2, POLR1C, PRKRR2, PRQCR, PSMC1, RABGAP1, RAD21, RAI14, RAN, RNASEN, RPF2, RPL21, RPL21P14, RPL23A, RPL28, RPL36A, RPS24, S100A9, SCEL, SGCE, SMARCA5, SMC4, SNCG, SNTB2, SPRR1A, SPRR1B, STAT3, STOM, SUV39H2, TBCE, TMSB10, TNS3, TOP2A, WDR12, XRN2, ZNF148</td>
</tr>
<tr>
<td><strong>Nucleus</strong> (n=64; p&lt;0.001)</td>
<td>ACTL6A, APOBEC3F, BOP1, CASP3, CCNA2, CCND1, CEBPG, CNOT7, CPSF3, CSTB, CTNNB1, DDX21, DFFB, EIF3E, ERCC2, FBXO5, FOXK1, FOXO1, FXR1, GRWD1, GTF2F2, GTPBP4, HIF1A, HMGA1, HMGB1, HNRNPH2, ING2, INTS1, INTS6, KLK6, KRT17, LOR, LYAR, MCL1, MCM4, MYC, NFIB, NMD3, NOP56, OASL, ORC6L, PHF2, POLR1C, PRKRR2, PSMC1, RAN, RNASEN, RPF2, RPL36A, RSR1C, S100A9, SMARCA5, STAT3, STK24, SYNCRIP, TAF2, TCEA1, TFDP1, TNS3, TOP2A, WDR12, XRN2, YAP1, ZNF148</td>
</tr>
<tr>
<td><strong>Protein folding</strong> (n=14; p=0.001)</td>
<td>CCT6A, CCT7, CCT8, CCT8P1, DNAJA1, FKBP11, FKBP1A, HSP90AA1, HSPD1, HSPE1, PDIA6, PPID, SEP15, TBCE</td>
</tr>
<tr>
<td><strong>Apoptosis</strong> (n=28; p=0.008)</td>
<td>BIRC2, CARD6, CASP3, CGB8, CKAP2, CSE1L, CTNNB1, DDIT4, DFFB, ERCC2, FAIM, FXR1, GJA1, GB, HSPD1P6, HSPE1, LGALS7B, MADD, MCL1, MYC, NET1, PDCD10, PEG10, RAD21, RTN4, SLT, TNFRSF6B, TOP2A</td>
</tr>
<tr>
<td><strong>Cell cycle</strong> (n=32; p=0.02)</td>
<td>ADCY3, AIF1, ANLN, CCNA2, CCN1, CDC8A, CKAP2, CKS2, CTNNB1, DTMYK, FBXO5, HILS, ILK, KIF11, LFNG, LRRCC1, MYC, NCA, PPP3CB, PSMC1, RABGAP1, RAD21, RAN, SESN3, SKP2, SMC4, SUV39H2, TAF2, TFDP1, UHRF1, ZAK, ZC3HC1</td>
</tr>
<tr>
<td><strong>Adhesion</strong> (n=14; p=0.02)</td>
<td>FZD8, WNT10B, DKK1, CXXC4, CAMK2B, FOSL1, WNT7A</td>
</tr>
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</table>
Table 2. LCN2 downregulated genes according to their annotated functions

<table>
<thead>
<tr>
<th>Regulatory process or pathway</th>
<th>Representative genes</th>
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</thead>
<tbody>
<tr>
<td><strong>Endoplasmic reticulum (n=43; p&lt;0.001)</strong></td>
<td>ALG13, AGR2, AIFM1, BCAP31, CYP2F1, CYP2J2, CYP4B1, CYP4F12, DHCR24, DNAJC10, DNAE1L1, EB, EPHX1, ERGIC1, ERN2, FAF2, FMO3, HAX1, HSD11B2, LMAN2, LPCAT4, LRP2, MAN1B1, METTL7A, MMGT1, PEX16, PGAP3, PGRMC1, PPP1R15A, PRKCSH, SEC22C, SEZ6L2, SGK1, SGPP2, SIL1, SLC27A5, SSR4, STIM1, STT3A, TRAPP1, UGT2B17, UGT2B7, VAMP7</td>
</tr>
<tr>
<td><strong>Membrane (n=44; p&lt;0.001)</strong></td>
<td>ABC4, ADCY6, AIFM1, ATP6V0E1, ATP6V1B1, CAMK2N1, CELSR3, CHPT1, CLEC2D, CLIC1, CUL5, CYP2F1, CYP2J2, CYP4B1, CYP4F12, DNAJC4, EB, EPHX1, FMO3, FOS, GBAS, GCNT3, GDI1, GRIN1, HSD11B2, LAMP2, MAN1B1, PGRMC1, PICK1, POMGnT1, PON3, PPAP2A, PTPRF, SCNN1B, SLC12A2, SLC1A3, SLC1A5, SLC26A6, STT3A, SYTL2, TSPAN15, TSPAN31, UGT2B17, VSIG2</td>
</tr>
<tr>
<td><strong>Mitochondria (n=56; p&lt;0.001)</strong></td>
<td>ACADM, ACO2, ACOT13, ACSF2, AFG3L2, AIFM1, ALDH18A1, ATP5G2, ATP5L, BCKDHA, BRP44L, CHCHD2, CKB, COX7B, CTSA, D2HGDH, DCI, DPYS2, ETBF, FIS1, FOXRED1, GATM, GBAS, GRAMD4, HAX1, HSD17B4, IDH2, ISOC2, MCCC2, MRPL20, MRPL41, MSRB2, MTP1, NDUF1, NDUF2, NDUF8, NIPSAP1, PCCB, PCK2, PICK1, PNKD, PRODH, PTS, SDH2, SIRT5, SLC1A3, SLC25A10, SLC25A23, SUOX, TAZ, TNFRSF19, TSC22D3, UCP2, UQCR10, UQCRB, UQCRH</td>
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<tr>
<td><strong>Apoptosis (n=36; p=0.004)</strong></td>
<td>AFG3L2, AIFM1, ANG, ATP6AP1, BCAP31, CASP4, DAPL1, DHCR24, DNAE2, FIS1, ERN2, GAS1, HPRT1, KLF11, KRT8P9, LTB, LYZ, MOAP1, MRPL41, MST4, MUC5AC, NGFRP1, NME3, OPTN, PPP1R15A, PYCARD, SGK1, SLC5A8, SLK, TAF9B, TBRG4, TICAM1, TNFRSF14, TNFRSF19, TNFSF10, TSC22D3</td>
</tr>
<tr>
<td>Regulatory process or pathway</td>
<td>Cell lines</td>
</tr>
<tr>
<td>--------------------------------------------------------</td>
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<tr>
<td>Epithelium development/differentiation</td>
<td>TβR</td>
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<tr>
<td>Cell motility</td>
<td>TβR</td>
</tr>
<tr>
<td>Response to hypoxia</td>
<td>TβR</td>
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<tr>
<td>Regulation of cell proliferation</td>
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<tr>
<td>Regulation of Wnt receptor signaling pathway</td>
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<tr>
<td>Cytokine-cytokine receptor interaction</td>
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<tr>
<td>Cell motility</td>
<td>TβR KRAS</td>
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<tr>
<td>MAPK signaling pathway</td>
<td>TβR KRAS</td>
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<td>Axon guidance</td>
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<tr>
<td>Angiogenesis</td>
<td>TβR KRAS</td>
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<tr>
<td>Extracellular region</td>
<td>TβR KRAS Smad4</td>
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</tbody>
</table>

Changes in copy number in the TβR cell lines were compared to the H6c7 cell line. Alterations in copy number were categorised based on gains, and were further examined using pathway and gene ontology classifications.
Table 4. **Pathway analysis of the copy number losses due to TGF-β resistance and KRAS<sup>G12V</sup> expression in the H6c7 cell line.**

<table>
<thead>
<tr>
<th>Regulatory process or pathway</th>
<th>Cell lines</th>
<th>Representative genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Regulation of cell cycle</strong></td>
<td>TβR</td>
<td>E2F2, BMP2, BRCC3, DLGAP5, SMAD6, SPHK1, CENPF, BRIP1, PKMYT1, BRCA2, BIRC5, GAS1, BRCA1, CDT1, CCNE2, PLK4, ID2, EPGN, ZWINT, FOXG1, IL1B, ID3, LTB</td>
</tr>
<tr>
<td><strong>Blood vessel development</strong></td>
<td>TβR</td>
<td>SLIT2, VEGFC, ID1, EPGN, TGM2, IL1B, SEMA3C, AMOT, LOX, MKL2, FGF1, THBS1, NR2F2, TNFAIP2, ANGPTL4</td>
</tr>
<tr>
<td><strong>DNA repair</strong></td>
<td>TβR</td>
<td>EXO1, KIF2, CLSPN, XRCC3, BRCC3, NUDT1, GIYD1, GIYD2, BRCA2, BRIP1, RAD54L, ESCO2, BRCA1, DCLRE1B, POLE2, RAD51L1, POLQ</td>
</tr>
<tr>
<td><strong>BMP signaling pathway</strong></td>
<td>TβR</td>
<td>BMP2, MSX1, ID1, SMAD6, FST, SMAD4</td>
</tr>
<tr>
<td><strong>Cell adhesion</strong></td>
<td>TβR</td>
<td>CLDN8, PLXNC1, KITLG, CUZD1, PCDHB12, CCL5, PCDHA3, PCDH1, BARX2, ITGB8, ITGB6, COL12A1, GPMB, SPON2, THBS1, FN1, FLRT3, FLRT2, COL4A3, ICAM4, PCDHB5, ITGA1, NID1, NID2, PCDH7, SSPN, PCDH18, TGFBI11</td>
</tr>
<tr>
<td><strong>ECM-receptor interaction</strong></td>
<td>TβR</td>
<td>COL4A4, ITGB8, ITGB6, ITGA1, THBS1, FN1</td>
</tr>
<tr>
<td><strong>Positive regulation of apoptosis</strong></td>
<td>TβR</td>
<td>TXNIP, COL4A3, ITGA1, BRCA2, BRCA1, MAP3K5, RASGRF2, CASP14, IFNB1, ALDH1A3, RIPK3, TGM2, IL1B, ID3, CASP1, IGFBP3, LTB</td>
</tr>
<tr>
<td><strong>Cell adhesion</strong></td>
<td>TβR KRAS</td>
<td>LAMA1, VAV3, SAA2, SAA1, CCDC80, TGM2, DLL1, CX3CL1, COL8A1, FGF1, DPP4, CLDN16, CD274, HLA-DMB, HLA-DRA, HLA-F</td>
</tr>
<tr>
<td><strong>Wnt receptor signaling pathway, calcium modulating pathway</strong></td>
<td>TβR KRAS</td>
<td>WNT10A, WNT4, WNT3A, ROR2</td>
</tr>
<tr>
<td><strong>Proteinaceous extracellular matrix</strong></td>
<td>TβR KRAS</td>
<td>COL4A4, COL4A3, CTHRC1, WNT10A, WNT3A, CHI3L1, CILP, CCDC80, SPOC1, COL5A3, ECM1, APLP1, LAMA1, WNT4, SFTPD, SERPINA1, SPON2, COL8A1, FGF1, MFAP4</td>
</tr>
<tr>
<td><strong>TGF-β signaling pathway</strong></td>
<td>TβR, TβR KRAS, TβR KRAS Smad4</td>
<td>BMP2, ID2, ID1, SMAD6, FST, SMAD4, ID3, THBS1</td>
</tr>
<tr>
<td><strong>Pathways in cancer</strong></td>
<td>TβR, TβR KRAS, TβR KRAS Smad4</td>
<td>COL4A4, E2F2, FZD8, FGF5, BMP2, SMAD4, KITLG, BRCA2, BIRC5, CCNE2, VEGFC, WNT4, TCEB2, FGF1, FN1</td>
</tr>
<tr>
<td><strong>Angiogenesis</strong></td>
<td>TβR KRAS Smad4</td>
<td>KCNMA1, SOCS3, EDN1, NR4A2</td>
</tr>
</tbody>
</table>

Changes in copy number in the TβR cell lines were compared to the H6c7 cell line. Alterations in copy number were categorised based on losses, and were further examined using pathway and gene ontology classifications.
Table 5. **Analysis of genes upregulated due to TGF-β resistance and KRAS<sup>G12V</sup> expression in the H6c7 cell line.**

<table>
<thead>
<tr>
<th>Regulatory process or pathway</th>
<th>Cell lines</th>
<th>Representative genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell motility</td>
<td>TβR</td>
<td>CTHRC1, SMO, IL6, CCK, S100P, FYN, PAX6, NEUROG2, DNAH1, CX3CL1</td>
</tr>
<tr>
<td>Extracellular region</td>
<td>TβR</td>
<td>VIP, CTHRC1, DCD, CCK, CRELD2, EPDR1, TIMP4, CX3CL1, GPC4, CALCBI, RSPO4, COL9A2, ST3GAL2, SERPINA1, SPATA6, MUC15, EB13, MUC1, IL6, LCN12, KRTDAP, IGF2, TCN1, NPTXR, INHBE, MFAP2, MFAP4</td>
</tr>
<tr>
<td>Positive regulation of cell differentiation</td>
<td>TβR</td>
<td>SMO, IL6, FOXA2, HOXA11, SOX2, PPARG, MAP1B, PAX6, EOMES, IGF2</td>
</tr>
<tr>
<td>Cell migration</td>
<td>TβR KRAS</td>
<td>PLAT, S100P, NDN, PODXL, S100A9, CCL5, CDH4, PLAUR, VEGFC, EPHA4, ID1, FOXX1, HBEGF, SEMA3B, POU4F1, SEMA3A, THBS1, FGF2, ENG, ETV4, CEACAM1, NR2F1, ARHGDIB</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>TβR KRAS</td>
<td>PLAT, CDX2, WT1, EDNRA, VEGFC, SH2D2A, ID1, THBS1, TNFAIP2, ENG, FGF2, CEACAM1, ANGPTL4</td>
</tr>
<tr>
<td>Wnt signaling pathway</td>
<td>TβR KRAS</td>
<td>FZD8, WNT10B, DKK1, CXXC4, CAMK2B, FOSL1, WNT7A</td>
</tr>
<tr>
<td>Jak-STAT signaling pathway</td>
<td>TβR KRAS</td>
<td>LIF, CSF3, CBLC, SPRY1, IL29, IL4R, SPRY4</td>
</tr>
<tr>
<td>Response to wounding</td>
<td>TβR KRAS</td>
<td>S100A8, CFB, EFEMP2, ITGB2, C1R, GPR68, C1S, PROC, S100A12, CXCL10, ALOX15, CD36, SAA2, SAA1, SERPINF2, ITGB6, SERPINA3, TFPI, VCAN, FN1</td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>TβR KRAS</td>
<td>NRXN2, IL32, L1CAM, ITGB2, PCDHB12, MCAM, PCDH18, AMIGO2, CD36, ITGB6, MSLN, VCAN, MFAP4, FN1</td>
</tr>
<tr>
<td>Proteinaceous extracellular matrix</td>
<td>TβR KRAS</td>
<td>MATN2, MATN3, MMP9, EFEMP2, CCDC80, MFAP2, VCAN, MFAP4, FN1</td>
</tr>
<tr>
<td>Regulation of actin cytoskeleton</td>
<td>TβR KRAS</td>
<td>FGFR1, ITGB6, ITGB2, MYH14, FGD3, FN1</td>
</tr>
</tbody>
</table>

Changes in gene expression were compared to the H6c7 cell line. Alterations in gene expression were examined using pathway and gene ontology classifications.
Table 6. **Analysis of genes downregulated due to TGF-β resistance and KRAS<sup>G12V</sup> expression in the H6c7 cell line.**

<table>
<thead>
<tr>
<th>Regulatory process or pathway</th>
<th>Cell lines</th>
<th>Representative genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secreted</td>
<td>TβR</td>
<td>FGF5, MMP28, CCL5, NETO1, CXCL10, WNT4, SAA2, SAA1, SFTPD, SPON2, DEFB1, ANGPTL4,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PNLIPRP3, COL4A4, ICAM4, CILP, CHI3L1, C10ORF99, NID1, SLT2, VEGFC, DKK1, CXCL14,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CST6, SERPINB2, IGFBP5</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>TβR</td>
<td>COL4A4, WNT4, SFTPD, CILP, NID1, MMP28, SPON2, ANGPTL4</td>
</tr>
<tr>
<td>Pathways in cancer</td>
<td>TβR</td>
<td>COL4A4, VEGFC, FGF5, WNT4, TCEB2, SMAD4, FGF1</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>TβR KRAS</td>
<td>COL4A4, HApLN3, ELN, CRTAP, CCDC80, SPOCK1, NID1, COL5A3, EMILIN2, SMOC2, LAMA1, WNT4,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ITGB8, SFTPD, COL12A1, MFAP2, COL1A1, MFAP4, MFAP5, FN1</td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>TβR KRAS</td>
<td>PCDHGA12, FLRT3, SVEP1, PTPRM, NRXN2, NID1, PCDHB12, EMILIN2, PCDH18, LAMA1, CD36,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ITGB8, MSLN, DSG1, COL12A1, MFAP4, CDH10, DPP4, FN1</td>
</tr>
<tr>
<td>Focal adhesion / ECM-receptor interaction</td>
<td>TβR KRAS</td>
<td>COL4A4, LAMA1, VAV3, ITGB8, COL1A1, COL5A3, MYLK, KDR, FN1</td>
</tr>
<tr>
<td>Positive regulation of apoptosis</td>
<td>TβR KRAS</td>
<td>VAV3, HOXA13, PML, FOXO3, ZBTB16, DAPK2, ITSN1, PLAG1, PRUNE2, APOE, CDKN2C, CASP14,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SST, IGFBP3</td>
</tr>
<tr>
<td>Pathways in cancer</td>
<td>TβR, TβR KRAS</td>
<td>COL4A4, E2F2, FGF5, WNT10A, WNT3A, SMAD4, PML, ZBTB16, BIRC3, DAPK2, GLI2, DAPK1,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LAMA1, WNT4, FGF1, CSF1R</td>
</tr>
<tr>
<td>MAPK signaling pathway</td>
<td>TβR KRAS Smad4</td>
<td>FOS, DUSP4, DUSP2, NR4A1, FGF12</td>
</tr>
<tr>
<td>Pathways in cancer</td>
<td>TβR KRAS Smad4</td>
<td>FOS, PTGS2, IL8, CYCS, FGF12</td>
</tr>
</tbody>
</table>

Changes in gene expression were compared to the H6c7 cell line. Alterations in gene expression were examined using pathway and gene ontology classifications.
References


